2003-06-01

MHC Class I Antigen Presentation is Regulated by the SUMO-Conjugating Enzyme UBC9: a Dissertation

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A Dissertation Presented By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences,

Worcester

in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY IN IMMUNOLOGY AND VIROLOGY

June 2003

MHC CLASS I ANTIGEN PRESENTATION IS REGULATED BY THE SUMO-CONJUGATING ENZYME UBC9

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June 11, 2003

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Parts of this dissertation gave been presented in the following publications

York IA, Mo AX, Lemerise K, Zeng W, Shen Y, Abraham CR, Saric T, Goldberg AL, Rock KL. (2003) The cytosolic endopeptidase, thimet oligopeptidase, destroys antigenic peptides and limits the extent of MHC class I antigen presentation. Immunity. 18(3): 429-40.

Shen YL, York IA, Rock KL (2003). MHC class I antigen presentation is regulated by ubiquitin conjugating enzyme UBC9. In preparation.

Acknowledgements

Here, I would sincerely thank my wife, Jian Ni, for her persistent support and encouragement, and my baby Jacqueline Ming Shen, who brought me so much happiness. Could I say that she also have given me huge amount of support?

I have too much thankfulness to my mentor, Dr. Kenneth L. Rock, for his kind guidance and support. He has been so deeply needed not only in my research but even in my life. What can I say to him? He has been far beyond the best mentor to me, the one I will remember forever, the memory he has brought to me worth being cherished forever.

I also want to thank Dr. Ian A. York. Since I came to Rock lab, I have been working together with Ian. He is the one from whom you can learn more than you want to. Feel so free to discuss my every tiny piece of data with him. Every time when I feel disappointed, I know from where to get encouragement. What a good experience. I do not know whether I can still find such a mentor-like partner in the future. Maybe I am too dependent.

Here I want to thank all the members in the Rock lab. No matter what I wanted to learn, no matter what problem I met, you guys were always there. I would give my thanks to all of you, thanks for all the happy memories with you.

At last, I will thank my committee member, Drs. Raymond M Welsh, Leslie J. Berg and Janet M. Stavnezer, for your suggestions and comments in my thesis research.

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Abbreviations

BH	Bleomycin hydrolase
cDNA	Complementary DNA
CO2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
GFP (EGFP)	(Enhanced) green fluorescent protein
H2O	Water
HCM	Hybridoma culture medium
HLA	Human leukocyte-associated antigen
HPLC	High Pressure Liquid Chromatography
HSP	Heat shock protein
IL-2	Interleukine-2
MACS	Magnetic bead activated cell sorting
MHC	Major Histocompatibility Complex
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
Ova	Ovalbumin
PBS	Phosphate buffered saline
PCR	Polychain reaction
PSA	Puromycine sensitive aminopeptidase
SDS	Sodium dodecyl sulfate
siRNA	Short-interfering RNA
SUMO	Small ubiquitin-like modifier
TAP	Transporter associated protein
TCA	Trichloroacetic acid
TE	Tris-EDTA
TFA	Trifluoroacetic acid
TOP	Thimet oligopeptidase
Tris	Tris(hydroxymethyl) aminomethane
UBC	Ubiquitin conjugating enzyme

ABSTRACT

CD8 T cells recognize complexes of MHC class I and peptide on the surface of target cells. MHC class I antigen presentation is a long pathway, in which proteins are degraded by proteasomes to generating oligopeptides, which may be further trimmed by aminopeptidases in the cytosol. Peptides are transported into the ER, where they may be further trimmed by ER lumenal aminopeptidases and bind to newly-synthesized MHC class I complexes. Proteins degraded by the proteasome are generally tagged with ubiquitin by a combination of ubiquitin-conjugating enzymes and ubiquitin ligases. UBC9 is one ubiquitin conjugating enzyme, which does not conjugate ubiquitin, but instead conjugates small ubiquitin-like molecules (SUMO) to target protein. UBC9 has been found to regulate the functions of many proteins in vivo, most importantly by modifying nuclear transportation and function. Curing my thesis work, I studied the function of UBC9 in MHC class I antigen presentation.

UBC9 over-expression in COS cells co-expressing ovalbumin markedly increased presentation SIINFEKL (the immunodominant epitope from ovalbumin in the context of H-$2K^b$), and UBC9 overexpression increased cell surface H-$2K^b$ in general, suggesting that Ubc9 increased MHC class I antigen presentation by increasing peptide supply.

UBC9 did not increase synthesis or degradation of ovalbumin. In transient transfection experiments, Ubc9 increased presentation of SIINFEKL precursors that did, and that did not, depend on proteasomes for processing, as well as SIINFEKL precursors

targeted to the ER, bypassing cytosolic processing altogether. However, a C-terminal extended precursor of SIINFEKL, which requires only proteasomal processing before presentation, was the most markedly affected by UBC9 overexpression. This suggested that UBC9 was affecting the pattern of cleavages made by proteasomes in ways that enhance the generation of the C-terminus of SIINFEKL. Because presentation of SIINFEKL itself (which requires no further proteolytic processing) was also enhanced, UBC9 must also affect steps in the class I pathway that occur after the generation of the mature epitopes. UBC9 did not affect the rate of peptide degradation in cytosolic extracts or in intact cells.

These findings suggested that UBC9 might have multiple effects on the MHC class I antigen presentation pathway. Immunofluorescent microscopy demonstrated that UBC9 increased the expression of the beta subunits of immunoproteasomes (LMP2, LMP7, and MECL1) as well as of TAP1 and tapasin. In contrast, UBC9 expression did not increase levels of calnexin, calreticulin, ERp57, or Protein disulfide isomerase (PDI). Similarly, levels of leucine aminopeptidase were not increased in UBC9-transfected cells. Therefore, UBC9 overexpression increases the levels of some but not all components of the class I pathway.

UBC9 overexpression increased protein levels of MECL1, LMP2 or LMP7 that were under the control of viral promoters, and levels of MECL1 mRNA were similar in control vector and UBC9 transfected cells. Therefore, UBC9 did not increase the level of expression of these subunits through increased transcription. Pulse-chase experiments showed that UBC9 overexpression reduced the degradation of MECL1. Therefore, UBC9

increases the levels of at least some of these components of the MHC class I antigen presentation pathway by increasing their stability.

To know the biological significance of UBC9 in MHC class I antigen presentation, I used small interfering RNA (siRNA) to knock down UBC9. Though UBC9 can be successfully knocked down by siRNA, the UBC9-negative cells became very sick, and were not suitable for the study of MHC class I antigen presentation.

There are three forms of SUMO molecules in mammalian cells: SUMO-1, SUMO-2 and SUMO-3. My study suggested that SUMO-2 may be involved in UBC9's regulation of MHC class I antigen presentation, since mutant SUMO-2 blocked UBC9's ability to increase H-2K^b-SIINFEKL levels on the cell surface after the cells were loaded with ovalbumin.

To further study the function of UBC9, I mutated the active amino acid Cys 93 of UBC9 to Ser (UBC9OH). Unexpectedly, this mutant form (UBC9OH) has very similar effects as wild-type UBC9, increasing K^b-SIINFEKL levels at the cells surface. This suggested that UBC9 protein regulates MHC class I antigen presentation pathway proteins by direct or indirect protein interaction, rather than (or as well as) by SUMO conjugation. Taking account of SUMO-2 results, I propose that wild-type UBC9 (either transfected or endogenous) conjugates SUMO-2 to its substrates, and then UBC9 (wild-type or mutant) interacts with its sumoylated targets, thus affecting protein functions.

I also studied heat shock protein Hsp27, which is known to be a substrate for UBC9 in vivo. Hsp27 is expressed in a variety of tissues in the absence of stress, and may regulate actin dynamics.

Hsp27 overexpression decreased generation of H-2K^b-SIINFEKL complexes from SIINFEKL precursors that did, and did not, require proteasomes for processing, or that were targeted to the ER. Hsp27 over-expression did not affect protein synthesis, and globally decreased cell surface H2-K^b and H2-D^b levels, but did not affect HLA-A0302 level. Hsp27 overexpression inhibits the presentation of ER-localized SIINFEKL. Taken together, my datasuggested that HSP27 may inhibit MHC class I antigen presentation by affecting MHC class I molecules itself rather than peptide supply.

After Hsp27 was eliminated with siRNA, the effects were very similar to those seen with Hsp27 overexpression. Levels of H-2K^b-SIINFEKL decreased, and overall cell surface H-2K^b and H-2D^b levels decreased. It is possible that when Hsp27 is over-expressed, it acts as a dominant negative form, conferring a similar phenotype to Hsp27 knockdown. These observations suggest that Hsp27 plays an important role in MHC class I antigen presentation.

CHAPTER I: INTRODUCTION

In the battle between the immune system and infectious agents, T cells screen MHC class I-peptide complexes on the cell surface. When T cells find foreign peptides bound to MHC class I molecules, they are activated, proliferate, and destroy the infected or mutant target cells. In the MHC class I antigen presentation pathway, proteins are degraded by proteasomes into oligopeptides that are transported into the ER. Many oligopeptides are too long for MHC class I binding and these oligopeptides generally can be further trimmed to appropriate size by aminopeptidases in the cytosol or ER. In the ER lumen, 8-10-residue long peptides bind to empty MHC class I heavy chain/ β_2-microglobulin complexes with the help of tapasin and chaperones, including GRP78, calnexin, calreticulin, and ERp57. These chaperones are also thought to help proper folding and assembly of MHC class I molecules and stablize empty MHC class I molecules. After binding peptide, complexes are then transported to the cell surface. T cell receptors (TCR) on CD8+ CTL recognize the MHC class I heavy chain/β2m/peptide complexes at the cell surface. Many factors are involved in regulating MHC class I antigen presentation, including cytokines such as interferon-γ (IFNγ).

1. The pathway of MHC class I antigen presentation.

1.1 Most peptides in cell are generated by proteasomes in the cytoplasm

It has long been known that most MHC class I-bound peptides are generated in the cytosol (although some peptides are generated in the ER (1)). For example, Townsend et al. expressed influenza virus haemagglutinin (HA) in the cytoplasm by deleting the ER leader signal sequence, and found that the HA epitope was still presented on MHC class I molecules (2, 3). However, the main protein processing machinery for the generation of MHC class I-bound peptides was a mystery for a very long time. Due to the abundance of proteases in the endosome/lysosome, it was initially thought that lysosomal proteases might be key players in generating MHC class I bound peptides. However, when inhibitors of the lysosomal proteases (such as weak bases, leupeptin or E64) were used to block proteolysis in endocytic compartments, MHC class I antigen presentation was not affected (4, 5). Therefore, unlike peptides that bind to MHC class II molecules, most MHC class I peptides must not be generated in endosomes or lysosomes.

Another major protease involved in protein degradation is the proteasome, an ATP-dependent protease complex in the cytosol. Proteasomes are responsible for degrading many proteins, especially ones that have been conjugated with poly-ubiquitin (which is a 76 amino acid-long protein (6) (see below). Two types of proteasomes can be purified from cells, the 20S and 26S complexes. The 20S complex is a 28 subunit particle, which forms a four ring structure (7). Seven homologous β-subunits compose the two inner rings, while another seven homologous α rings make up the two outer rings. Basically the two inner rings are responsive for proteolysis, in which three of the β-subunits (β1, β2, and β5) are most important (8). The 26S proteasome is a very large particle composed of 20S (650-kDa) proteasome and two 19S (700-kDa) regulatory complexs, which are located at each

end of the 20S core particle (9). The 26S complex requires ATP to degrade proteins and is believed to be the principal form of the particle which degrades proteins in vivo. This larger complex is the only one that can degrade poly-ubiquitinated proteins.

The discovery of the proteasome components LMP2 and LMP7 in the MHC class II region of the genome suggested a role for proteasomes in MHC class I antigen presentation (10-13). Using cells that exhibit a temperature-sensitive defect in ubiquitin conjugation, Michalek et al. found that at the non-permissive temperature MHC class I-restricted presentation of ovalbumin introduced into the cytosol was inhibited, but presentation of an ovalbumin peptide synthesized from a minigene, which does not require proteolysis for presentation, was not affected. Since proteasomes are required for polyubiquitinated protein degradation, this finding suggested that proteasomes are required for MHC class I antigen presentation (14). Another line of evidence showing that the proteasome is involved in generating peptides for antigen presentation involved altering the rate of degradation of an antigenic substrate by the proteasome. Protein stability is affected by the N-end rule, in which its N-terminal amino acid affects the rate of ubiquitination and degradation by proteasomes (15). By modifying beta-galactosidase with a destabilizing amino-terminal residue, Grant et al. found that the rate of MHC class I presentation of a β-gal derived peptide was enhanced. This enhanced presentation was inhibited by blocking potential ubiquitination sites on the protein through methylation of epsilon amino groups, and by peptide aldehyde inhibitors of the proteasome (16). Yet another line of evidence for the role of the proteasome in antigen presentation involved the use of proteasome inhibitors. When cells were treated with these agents, both protein degradation and the generation of most

presented peptides was blocked (5, 17). Therefore, proteasomes catalyze the degradation of the vast majority of cell proteins and generate most peptides presented on MHC class I molecules.

1.2 The main source for generating MHC class I bound peptides: DRiPs or native proteins?

It was originally thought that class I-presented peptides were derived from the degradation of full length proteins. However, recent work has suggested that defective ribosomal products (DRiPs) may be the main source of peptides to MHC class I molecules (18). According to this hypothesis, many newly synthesized polypeptides never attain their native structures owing to errors in translation or in post-translational processes necessary for proper protein folding (19-21). These incomplete or abnormal proteins are rapidly degraded by the proteasome, generating the class I-presented peptides. A more recent study also suggested that newly made proteins are required for MHC class I antigen presentation for long-lived proteins (22). In their work, they utilized lymphocytic choriomeningitis virus (LCMV) nucleoprotein, which has a half-life of >3 days, as a model protein. Two days after nucleoprotein expression was induced, they terminated protein expression, and found that the cells rapidly ceased antigen presentation, although the protein was still present and presumably being degraded by proteasomes. This result supported the DRiPs theory. This process might be advantageous as a mechanism to insure rapid MHC class I-restricted CTL responses after cell mutation or infection. Although all proteins are continuously degraded, there might be a delay between the synthesis and degradation/presentation of relatively

stable proteins during which time pathogens could multiply. Although this theory may explain how many peptides are generated for MHC class I molecules, this cannot be the whole story. When mature ovalbumin protein is introduced directly into the cytoplasm of cells by the osmotic lysis of pinosomes, it is degraded and its epitopes are presented by MHC class I molecules (3). In our experiments, when cells are directly loaded with full length ovalbumin antigen (which is rapidly degraded), we can detect H2-K^b-SIINFEKL complexes on cell surface in only half an hour. Since in this system there is no synthesis of ovalbumin, protein synthesis is clearly not absolutely linked to antigen presentation. The N-end rule also suggested that the protein degradation rate of newly synthesized mature proteins is crucial for peptide generation (because DRiPs are presumed to all be very rapidly degraded) (23, 24). MHC class I presentation can be inhibited by blocking potential ubiquitination sites on the protein through methylation of amino groups (16). So it is not presently known how important or in what situations DRiPs play a role in generating presented peptides.

1.3.Proteasome composition is changed in IFNγ-treated cells.

It has long been known that IFNγ treatment up-regulates MHC class I antigen presentation. INF-g increases the expression of most of the known components of the class I pathway, including the class I heavy and light chain and the TAP transporter. In addition, three proteasome subunits, LMP2 (MB1), LMP7 (δ) and MECL-1 (LMP10), have been found to be induced by IFNγ and to replace, respectively, the constitutive proteasome subunits β1, β2, and β5 (25, 26). The resulting particle is called the "immunoproteasome"

(27). Immunoproteasomes and constitutive proteasomes have very different proteolytic activity and often result in different cleavage patterns on the same antigen (28, 29). LMP2 and LMP7 are encoded in the mammalian MHC locus (30, 31). Early experiments showed that LMP2 and LMP7 were not essential for generating peptides for MHC class I antigen presentation (10). Subsequently, however, it was found that LMP gene products specifically alter the peptidase activities of the proteasome, which in turn alters the peptide profiles on MHC class I molecules (12, 13). This is because these 3 immunoproteasome subunits encode the active proteasome's active sites. Both LMP2 and LMP7 knockout mice and cells lacking these subunits showed decreased rates of cleavage after hydrophobic and basic residues and increased hydrolysis after acidic ones. Overexpression of LMP2 and LMP7 had the opposite effects. This change in cleavages should generate more peptides that end in hydrophobic and basic residues, which are the precisely the kind of peptides found on MHC class I molecules (32, 33). Loss of LMP2 or LMP7 reduces the presentation of certain epitopes. In contrast, overexpression of LMP2, LMP7 and MECL-1 has been found to increase presentation of the LCMV immunodominant epitope (34).

Recent findings suggested that the incorporation of IFNγ inducible proteasome subunits not always enhance antigen presentation, but also may reduce the generation of certain CTL epitopes (35). For example, Morel et al. found that an EBV antigen, RU1, cannot be processed by immunoproteasomes but can be processed by standard proteasomes (27, 36). It seems that immature, but not mature, dendritic cells can present the RU1 epitope to CTL, which is consistent with the finding that mature DC express only immunoproteasomes while immature DC express both standard and immunoproteasomes (37, 38).

IFNγ treatment affects other associated components of the proteasome in addition to the catalytic beta subunits. An 11S heterohexameric or heteroheptameric complex, which is composed of two homologous subunits, PA28α and PA28β, is a regulatory complex of the 20S proteasome. Both PA28α and PA28β subunits are markedly increased upon IFNγ treatment, suggesting its involvement in antigen processing and MHC class I antigen presentation. Overexpression of PA28α in a mouse fibroblast line expressing the murine cytomegalovirus pp89 protein has been found to markedly enhance its recognition by pp89-specific cytotoxic T cells (39). Also, mice deficient in PA28β gene have defects in antigen presentation (40). In one study, the assembly of immunoproteasomes (containing LMP2, LMP7 and MECL-1) was greatly impaired in these PA28β deficient mice, suggesting that PA28 was required for immunoproteasome assembly. In contrast to the findings with the PA28β knockout mice, in PA28α/PA28β double knockout mice immunoproteasomes were induced upon IFNγ treatment (41), possibly suggesting that loss of PA28α itself may impair immunoproteasome assembly. Although immunoproteasomes are assembled in PA28α/PA28β double knockout mice, these mice have defects in processing a melanoma antigen TRP2, but not in processing ovalbumin or influenza A virus, suggesting that PA28α/PA28β complexes affect the processing of certain antigens but not others.

1.4. Cytosolic proteases are involved in peptide trimming

To fit into the MHC class I molecule peptide-binding cleft, peptides must be 8-10 residues in length (8, 42). In vitro experiments results suggest that mammalian 20S and 26S

proteasomes generate peptide products ranging from 3 to 22 amino acids (43) during degradation of different proteins (44, 45). Using ovalbumin as a model protein, Cascio et al. found that 26S and 20S proteasomes generated, as well as products shorter than 8 amino acids, SIINFEKL itself as well as peptides containing 1-7 extra N-terminal residues preceding SIINFEKL, while immunoproteasomes released 2-4 times more of certain N-extended versions, but no more of the correct-sized peptide SIINFEKL (29). These N-extended peptides would have to be further processed for tight binding to MHC molecules, or else just ignored by antigen presentation.

Much work has been done in our lab and others to study whether proteasomes are necessary in trimming longer peptides to the correct size. When minigenes encoding SIINFEKL peptides with several extra N-terminal residues were introduced into cells, SIINFEKL can be efficiently presented by H-2K^b cells in the presence of proteasome inhibitors. However, if even one extra residue exists at the C-terminal of peptides, SIINFEKL presentation is inhibited by proteasome inhibitors (46, 47). In addition, acetylation of the amino terminus of an extended construct, which prevents trimming by aminopeptidases (but not endopeptidases), prevents the presentation of these peptides (47). These results suggest that the C terminus of antigenic peptides must be generated by the proteasome, but that other proteases, particularly aminopeptidases, can also generate the N-termini.

The first aminopeptidase specifically implicated in antigen processing was leucine aminopeptidase (LAP) (48). N-terminal trimming was found to be several-fold faster in

cytosolic extracts of IFN-γ treated cells then that of untreated cells, and the IFN-inducible activity was found to be LAP. Purified LAP quickly trimmed the 11-mer QLESIINFEKL peptide to SIINFEKL in vitro. Very interestingly, LAP expression is enhanced by IFN-γ treatment, suggesting that this aminopeptidase is involved in peptide trimming and MHC class I antigen presentation. More recently, puromycin-sensitive aminopeptidase (PSA) and bleomycin hydrolase (BH) have also been found implicated in peptide trimming. These two proteases could remove NH_2-terminal amino acids from the vesicular stomatitis virus nucleoprotein cytotoxic T cell epitope 52-59 (RGYVYQGL) in cells not treated with IFN-g (49). Besides these cytosolic aminopeptidases, an ER lumen-resident aminopeptidase (ERAP1) has recently been found, and this will be discussed in detail below.

1.5 TAP transports peptides into ER

Most peptides for MHC class I antigen presentation are generated in the cytosol, and then transported into ER, where they bind to empty MHC class I molecules. Definitive evidence that these peptides are generated in the cytoplasm came from the analysis of mutant cells lacking the transporter associated with antigen processing (TAP). TAP deficient human cell line LBL 721.174 and murine cell line RMA-S have profound defects in MHC class I antigen presentation (50, 51), and only efficiently present peptides that are targeted to the ER through signal sequences (52). Similarly, in TAP-deficient mice, stable MHC class I molecule assembly is defective and cell surface MHC class I levels are markedly reduced due to loss of peptide supply from the cytoplasm (53-55).

TAP is an ER membrane-integral heterodimer. Both of its subunits, TAP-1 and TAP-2, are encoded by genes in the MHC class II locus, closely linked to the LMP2 and LMP7 genes (56, 57). They are ATP-binding cassette (ABC) transporters capable of translocating peptides from the cytosol to in the lumen of the ER, where MHC class I molecules are waiting. Human TAP1 and TAP2 exhibit 64% homology at the protein level, and they have a similar predicted membrane topology. There are eight and seven transmembrane (TM) segments for TAP1 and TAP2, respectively. TAP1 has both its N and C terminus in the cytoplasm, whereas TAP2 has its N terminus in the lumen of the ER. TAP is composed of three sub-domains: a TM pore, a cytoplasmic peptide-binding pocket, and a nucleotide-binding domain (NBD) (58)

Peptide transportation by TAP involves several steps. The first step is peptide binding to TAP, which is an ATP-dependent process. Peptide binding causes topological changes in the TAP complex, which trigger ATP hydrolysis and peptide translocation across the membrane (59, 60). Recently, Cresswell and his colleagues found that ATP hydrolysis by TAP2, but not TAP1, is needed for both peptide binding and transportation (61). As with many other components of the MHC class I antigen presentation machinery, TAP expression is induced by IFNγ.

Several studies have characterized the substrate specificities of TAP (62, 63). Based on peptide trapping experiments, random peptide mixtures of 9-16 amino acids displayed significantly higher affinity for the binding site than mixtures of shorter or longer peptides (59). TAP also has sequence preferences. The N-terminal three positions and the C-terminal

residue are much more critical than other peptide positions (64). Mouse TAP has a strong preference for peptides with hydrophobic C-terminal amino acids, while human TAP also binds peptides with basic C-termini (65, 66), which in each case correlates with the preference of the MHC molecules of each species. This also correlates with the kinds of peptides generated by immunoproteasomes (see above). TAP polymorphism does not measurably alter its specificity for peptide substrates (67).

1.6 Missing steps between peptide generation and TAP transportation.

Present data show that proteasomes in the cytoplasm generate short peptides that are transported by TAP into the lumen of the ER, where the transported peptides bind to empty MHC class I molecules. Are there any missing steps between proteasome and TAP? One remaining question is how proteasome-generated peptides reach TAP molecules. It has been proposed that proteasomes sit on the outer membrane of the ER, so that peptides can be transported into ER by TAP as soon as the peptides are generated, or that there are carrier molecules that help to transport peptides to TAP.

One possibility is that, for efficient peptide binding to MHC class I molecules, there is a physical association between immunoproteasomes, TAP1/2 complexes and MHC class I molecules (68). If so, the MHC class I protein processing and presentation machinery could be regulated not only by the level of expression of its components, but also by their localization within the cell in ways that facilitate peptide binding and MHC class I antigen presentation. If this suggestion is correct, then if the proteasome or immunoproteasome

generates a longer than authentic MHC class I binding peptide, it would be immediately transported into the ER, bypassing the cytosol. Consequently cytosolic enzymes would not play any role in trimming or degradation of epitopes. Since cytoplasmic aminopeptidases, such as leucine aminopeptidase, puromycin-sensitive aminopeptidase and bleomycin hydrolase, have been shown to be involved in MHC class I antigen presentation (48, 49), this model is probably incorrect. Also, overexpression of a cytoplasmic endopeptidase, thimet oligopeptidase (TOP), strongly decreases MHC class I antigen presentation (69), showing that peptides are accessible to cytosolic enzymes.

Another possibility is that there are carrier proteins between proteasomes and TAP1/TAP2 complexes. It has long been proposed that peptides exist in the cytosol and ER in association with heat shock proteins (70). HSPs are now known to play diverse roles, even in unstressed cells, in protein folding, assembly, intracellular localization, secretion, regulation, and degradation of other proteins (71-73). HSPs may also play some roles in immune response (74), especially in anti-tumor immune response (75-78).

Indeed it has been found that HSPs, such as HSP70 molecules and the ER-resident glucose-regulated protein 94 (grp94) bind peptides in vivo, though their role in MHC class I antigen presentation is not clear. Injection of HSP-bound peptides into animals has been found to elicit CTL responses to the bound peptides (79, 80). After conditional overexpression of HSP70 in a human melanoma cell line, Dressel et al. found that HSP70 does not affect MHC class I cell surface expression but did increase killing by CTL (81), suggesting that HSP70 may facilitate transportation and presentation of certain peptides.

Using a novel assay, Shastri's group recently found that N-terminally extended antigenic analogs were associated with high–molecular weight (30–60 kDa) material in the cytosol before transport into ER through TAP (82). It is not known what these molecules are , and more definitive studies need to be done, but this finding again raises the possibility that carriers may exist between peptides generated by the proteasome and transportation by TAP. Recently, Neefje's group found chromatin/histones can bind peptides and protect them from degradation (83).

Another HSP, the 27-kDa heat shock protein (HSP27), which is expressed in a variety of tissues in the absence of stress, is a potentially significant effector protein that may regulate actin dynamics, possibly by a phosphorylation/dephosphorylation mechanism (84). HSP27 is involved not only in apoptosis, such as virus-induced cell death (85), but also in cell survival, such as injured sensory and motor neuron survival and TNF-mediated apoptosis (86, 87). Human HSP27 is a potent inducer of IL-10 in human monocytes, suggesting that it may have a role in immune response (88). It has not been reported that HSP27 is involved in MHC class I antigen presentation, however, I will show some data, in which altering its expression can influence MHC class I antigen presentation.

1.7.Aminopeptidases may trim longer peptides to the correct size in ER lumen

Proteasomes generate N-extended precursors of antigenic peptides that can be trimmed by aminopeptidases to the correct size for presentation. It has been shown that longer

peptides could be trimmed by aminopeptidases in the ER, as well as in the cytosol (89). It has been demonstrated that an ER aminopeptidase termed ERAP1 or ERAAP is at least one of these ER aminopeptidases, for if ERAP1 is knocked out, the presentation of some longer ER peptides is inhibited (1, 90, 91).

1.8. ER chaperones facilitate MHC class I complex assembly

To ensure that only peptide-loaded MHC class I complexes are transported to the cell surface, empty MHC class I complexes may be retained in the ER by a set of ER resident chaperones, including calnexin and calreticulin (92), and ERp57 (93). The ER chaperones help to control the quality of the exported MHC class I molecules by helping MHC class I proper folding and assembly. The unloaded or misfolded MHC class I molecules are transported to the cytosol, where they are degraded by proteasomes (94).

Calnexin associates with newly synthesized free MHC class I heavy chains and, in mouse but not human cells, newly assembled class I heavy chain–$ß_2$m dimers (95, 96). Calreticulin binds MHC molecules later than calnexin, and remains associated with assembled MHC class I molecules (97) until the complex binds peptides and is transported out of the ER. Both calnexin and calreticulin interact with MHC molecules by binding monoglucosylated N-linked glycans.

Another important ER protein involved in MHC class I antigen presentation is tapasin, which is a transmembrane protein that tethers empty MHC class I molecules to TAP

complexes (98, 99) and also enhances peptide loading of MHC class I complexes in some other way. Tapasin retains MHC class I molecules in the ER until they acquire high-affinity peptides. Tapasin knockout mice show reduced MHC class I antigen presentation, a skewed repertoire of peptides displayed on the cell surface, and a poor antiviral immune response (100, 101).

Yet another MHC class I associated protein in the ER is ERp57, a thiol oxidoreductase. It may be involved in disulfide bond isomerization of MHC class I molecules in peptide loading (102). Since no ERp57 knockout mice are available now, it is not known whether ERp57 is critical for MHC class I antigen presentation. Our lab has used siRNA to eliminate ERp57 in several cell lines, but we did not find a significant reduction in MHC class I antigen presentation (I.A.York et al., unpublished data).

1.9. Some pathogens can evade the MHC class I antigen presentation pathway

MHC class I antigen presentation is a complicated pathway, involving many components and steps. Many viruses, including herpesviruses, HIV, and adenovirus, have been found to evade immune response by blocking MHC class I antigen presentation (103-109). Many steps in the class I pathway can be inhibited by viral proteins after infection. For example, herpes simplex virus ICP47 protein inhibits MHC class I antigen presentation by binding to the TAP complex in ways that block peptide transport (110, 111). Studying viral immune evasion may provide us with more insights into MHC class I antigen presentation.

2 . Ubiquitin-conjugating enzymes are involved in protein degradation

In the last three decades, it has been found that, under most conditions, the ubiquitin-proteasome pathway is responsible for the lion's share of selective degradation of cellular proteins (112). Ubiquitin is a small 76 amino acid protein with many roles in vivo. Monoubiquitination is found to function as a signal in activities ranging from endocytosis (113, 114) to transcription regulation (115), which will not be discussed in this introduction. Polyubiquitination is used by cells to target proteins for degradation by the proteasome.

In the process of polyubiquitination, there is a series of three kinds of enzymes involved: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin protein ligase (E3). In this process, with ATP providing energy, ubiquitin binds to a cysteine residue on E1 via a thioester bond. This activated ubiquitin is then transferred to a cysteine residue on one of the many E2 molecules. In the presence of E3 molecules, E2 molecules transfer ubiquitin to the ε-amino group on lysines of target protein, forming an isopeptide bond. By repeating this process, further ubiquitin molecules will be transferred to the ε-amino group of lysine of the existing ubiquitin, generating polyubiquitinated proteins (6).

Although there is only one E1 molecule in mammalian cells, more than 20 E2 have been found so far (116). It is believed that any one of E2s may serve several E3s. The different combinations of E2 and E3 molecules may target ubiquitin to specific proteins for

degradation. In most cases, E3s may be regulated or modified by additional molecules, which may confine the specificity of the target proteins (117, 118).

Recently, it was found that polyubiquitination was involved in not only in the destruction of soluble cellular proteins, but also in degradation of membrane-bound proteins. Indeed, ER proteins have been found to be transported to the cytosol to be degraded (119-121). This may also contribute peptide supply for MHC class I antigen presentation, especially of ER-targeted proteins (121, 122). Retrotranslocation of proteins from the ER to the cytosol requires energy, perhaps because ubiqutination and/or the proteasome is involved.

There are many excellent papers that review the ubiquitin-proteasome system (112, 116). Here, I will discuss the function of one of the E2 molecules, UBC9, which is not involved in protein degradation, but which may modify the function of target proteins.

3 Ubiquitin conjugating enzyme (Ubc) 9

3.1 UBC9 is an E2-like molecule

UBC9 was first found in yeast as a nuclear protein involved in cyclin degradation (123). Mutation of yeast UBC9 prevented cell cycle progression at G or early M phases. Shortly thereafter, UBC9 homologues were cloned from human cells and mouse cells (they

are identical at protein level (124, 125)), and were found to be associated with several nuclear proteins such as RAD51, p53, cyclin (126).

Many studies have attempted to elucidate the function of UBC9, and its exact roles and mechanisms of action are still not well understood. It has been observed to have many different effects in cells. A limited number of studies have found a role in protein degradation. Haber and his collaborators found that UBC9 was involved in the degradation of E2A, a helix-loop-helix protein involved in cellular growth and differentiation(127, 128). Tashiro et al. found that UBC9 was directly involved in proteasome-dependent proteolysis of I-κBα (129). Therefore, it was proposed that, as with other UBCs, UBC9 is involved in the polyubiquitination and proteasome-dependent degradation of these nuclear proteins. However, shortly thereafter, it was found that UBC9 does not conjugate ubiquitin to target proteins. Rather, it conjugates SUMO-1 (small-ubiquitin-related modifier) (130)) to substrates (131, 132), suggesting that UBC9 may be not involved in ubiquitin-mediated proteasome-dependent protein degradation. In fact, further studies, described below suggest that UBC9 generally does not play a role in protein degradation.

SUMO-1 has many different names such as PIC1 (133), sentrin (134), GMP1 (135), Ubl1 (136), Smt3c (137), and hSmt3(138), for it was found by different groups within a few months in S. cerevisiae, and mice, and human, respectively. As well, two related proteins, SUMO-2/SMT3A (139) and SUMO-3/SMT3B (137) have been characterized, and are also conjugated to target proteins by UBC9 (140).

Although SUMO-1 and ubiquitin have very similar structures (141), they share only 18% amino acid sequence identity. The first protein found to be conjugated to SUMO-1 was Ran GTPase–activating protein (RanGAP1) (130). It was also found that UBC9 is required for SUMO-1 modification of RanGAP1 (142-144). Mammalian RanGAP1 is concentrated at the cytoplasmic face of the nuclear pore complex (NPC), and is involved in nuclear protein import. Interestingly, only SUMO-1-conjugated RanGAP1 is associated with the nuclear protein RanBP2, which is involved in protein import, suggesting that sumoylation may be important in nuclear protein translocation. In mitosis, SUMO-1 conjugation is required for RanGAP1 to associate with the mitotic spindle, suggesting that sumoylation plays an important role in spatial regulation of the Ran pathway during mitosis (145). Also, sumoylation with SUMO-1 re-localized Homeodomain-interacting protein kinase 2 (HIPK2) to nuclear speckles (146). These results suggest that UBC9 may be not involved in protein degradation, but may instead modify the function or localization of its substrate proteins.

Another function of UBC9 is to regulate the activity of transcription factors. Since UBC9 is a nuclear protein, it is not surprising that it can modify transcription factors. A sumoylation motif can be found in many steroid receptors, suggesting that UBC9 might play a role in the regulation of transcription. In particular, UBC9 interacts with the androgen receptor (AR), a member of the steroid receptor family, and activates receptor-dependent transcription (147), and AR can be covalently modified by SUMO-1 in an androgen-enhanced fashion (148). Another well-studied transcription factor modified by UBC9 and SUMO-1 is p53. p53 trans-activation ability is increased by SUMO-1 modification (149,

150). Sumoylation does not cause p53 degradation, but inhibits the degradative pathway and increases the activity of p53 as a consequence. The sumoylation site on p53 is K386, which is not utilized by ubiquitin. UBC9 has been found to increase the activity of NF-κB by associating with its upstream regulating protein MEKK1 and the type I TNF-alpha receptor (151) and by degrading I-κBα, which is a NF-κB inhibitor (129). Other nuclear factors modified by SUMO-1 include Mdm2 (152, 153), C-Jun (154), topoisomerase (155-157), AP2 (158), and many other transcription factors (159).

In fact, UBC9 does not only regulate the activity of nuclear factors. UBC9 also binds glucose transporter GLUT1 and GLUT4 and regulates their function (160). When UBC9 is over-expressed in L6 skeletal muscle cells, the basal glucose transport by GLUT1 transporter decreased 65%. By contrast, GLUT4 transport is increased about 8 times, leading to enhanced transport stimulation by insulin. The mechanism by which UBC9 regulates GLUT1 and GLUT4 is not known yet.

Although it has been reported that that UBC9 is involved in protein polyubiquitination and degradatio, e.g. for I-κBα and E2A, (127-129), this may be not in fact be valid or generally true. Hay and his colleagues found that UBC9 conjugates SUMO-1 to I-κBα, and the sumoylated I-κBα is resistant to proteolysis (161). This is consistent with the finding that UBC9 conjugates SUMO-1 but not ubiquitin to target proteins. UBC9 was also found to conjugate SUMO-1 to K21 of I-κBα, which is also utilized for ubiquitin modification. Unlike polyubiquitination of I-κBα, which is phosphorylation-dependent, phosphorylation of S32 and S36 of I-κBα inhibits SUMO-1 modification (161, 162). In this case, it seems

that sumo blocks the ubiquitination site and thereby prevents polyubiquitination and degradation. Another HLH transcription factor, TEL, was modified by SUMO-1 (163), and also interacts with UBC9 through its HLH domain, but this interaction did not lead TEL degradation (164). Whether E2A is like I-κBα, in which ubiquitin and sumo utilize the same motifs, should be further explored.

3.2. Sumoylation is an enzymatic process very similar to ubiquitination

SUMO-1 is conjugated to target substrates by a mechanism similar to ubiquitin. The SUMO-1 activating enzyme contains two subunits, SAE1/SAE2 (165). In vitro, in the presence of ATP and recombinant SAE1/SAE2, a thioester linkage can be generated between SUMO-1 and SAE2. If UBC9 is present, SUMO-1 is transferred to UBC9 from SAE2. When the SUMO-1 substrate I-κBα is added, SUMO-1 was efficiently transferred to I-κBα from UBC9. SUMO-2 and SUMO-3 also use SAE1/SAE2 for sumoylation in vitro (140). As with some ubiquitin-conjugating enzymes, UBC9 can directly sumoylate substrates in vitro without the help of E3-like enzymes. However, due to the huge variety of proteins in cells, and the specificity of sumoylation, it is very unlikely that UBC9 conjugates SUMO-1 molecules to substrates without the help of E3-like molecules.

Recently, E3-like molecules have been described in the sumoylation pathway. The first E3-like molecules, Siz1 and Siz2, were found in S. cerevisiae (166, 167). It was found that these two molecules were required in most known SUMO-1 conjugations in yeast. However, it seems unlikely that UBC9 and Siz1/Siz2 can specifically promote SUMO

conjugation into many substrates, although the authors argued that Siz1/Siz2 promoted SUMO conjugation to different substrates at different stages of the cell cycle. Many SUMO E3-like molecule have been found in mammalian cells, including PIAS1 (159, 168), PIASy (169), RanBP2 (170, 171).

Additional molecules, such as E4, may help to provide specificity to the conjugation of ubiquitin to substrates (117). Since sumoylation is so similar to polyubiquitination, it would be not surprising if E4-like molecules are found in the future to participate in sumoylation.

In mammalian cells, there are at least three SUMO-like molecules: SUMO-1, and two highly homologue molecules, SUMO-2 and SUMO-3. Although it has been found that SUMO-2 and 3 were more abundant than SUMO-1 in COS cells (172), their functions are less studied. Under normal conditions, most SUMO-2 and SUMO-3 molecules exist in the free form. Interestingly, SUMO-2/3 were conjugated to high-molecular-mass proteins when the cells were subjected to protein-damaging stimuli. Also, it seems that SUMO-2 and 3 do not conjugate to SUMO-1 substrates, such as RanGAP1. SUMO-2 and 3 also differ from SUMO-1 in that they can form polymeric chains in the absence of substrate in vitro, while SUMO-1 can only form monomers on its substrates. However, it is not known whether SUMO-2 and 3 polymeric chains are formed in vivo. In fact, it is unknown how physiologically important SUMO-2 and 3 are in vivo. Nevertheless, these results suggest that SUMO-2 and 3 have different functions from SUMO-1.

An interesting finding is that mutation of the conserved active amino acid Cys to Ser (C93S) of UBC9, which prevents sumoylation by disrupting the formation of a thiolester bond between SUMO-1 and UBC9, blocks UBC9's sumolylating activity but has no effect on its function in stimulating AR-dependent transactivation. This may suggest that UBC9 can act as an AR coregulator independent of sumoylation. AR is not exceptional in its being affected by mutant UBC9. Many papers have showed that mutation of Cys 93 in UBC9 does not affect its interaction with substrate proteins (164, 173, 174), even though most of the interacting proteins are SUMO-1 substrates (163). How does mutant UBC9 regulate substrate protein functions? One explanation is that UBC9 can regulate the function of its substrates by direct binding in the absence of SUMO molecules. Another possibility is that since most of these studies have been done by overexpressing mutant UBC9 in cells that constitutively express wild type UBC9, the mutant UBC9 might modify the function of its substrates by binding to a previously conjugated SUMO molecule. In other words, in this model mutant UBC9 binds to its substrates only after endogenous (non-mutated) UBC9 has conjugated SUMO to target proteins.

4. Object of this project

UBC9 is involved in MHC class I antigen presentation

In our study of how different UBC molecules regulated protein degradation and MHC class I antigen presentation, we found that UBC9 overexpression increased Kb-SIINFEKL level on COS-Kb cell surface when ovalbumin is used as model protein. The goal of this

thesis was to investigate the basis for this effect and to elucidate the potential role of UBC9 in antigen presentation.

CHAPTER II MATERIALS AND METHODS

Cell lines

Cell lines used in this experiment include COS-7 cells (African Green Monkey kidney cells) (175); COS-K^b (H-$2K^b$ transfected COS-7 cell line) (1, 91); Hela (176, 177); Hela-K^b (H-$2K^b$ transfected Hela cell line) (1, 91) and E36-K^b cell (E36.12.4) (E36 transfected with H-$2K^b$ and ICAM) (47). COS-7 cells were grown in complete RPMI1640 medium (RPMI1640 medium supplemented with 10% Fetal Bovine Serum (FBS), L-Glutamine, penicillin (100U/ml), streptomycin (100U/ml)). COS-K^b and E36-K^b cell lines were maintained in complete RPMI1640 medium supplemented with G418 (250μg/ml). Hela cells were maintained in complete DMEM medium (DMEM medium supplemented with 10% FBS L-Glutamine, penicillin (100U/ml), streptomycin (100U/ml)). Hela-K^b cells were maintained in complete DMEM medium supplemented with G418 (250μg/ml).

Antibodies

The H-$2K^b$-SIINFEKL specific antibody 25.D1.16 (178) was kindly provided by Dr. R. Germain (NIH, Bethesda, MD). W6/32 (179) is specific for HLA-A, B, C; Y3 (180) is specific for H-$2K^b$. 12CA5 recognizes the HA tag (181, 182). Rabbit anti-MECL1, anti-LMP2, and anti-LMP7 (183-185), were kindly provided by Dr. A.L. Goldberg, Harvard Medical School, and anti-PA28α and anti-PA28β (186) were kindly provided by Dr. George DeMartino (University of Texas Southwestern Medical Center, Texas). Anti-UBC9 monoclonal antibody was purchased from BD Pharmingen (San Diego, CA). Rabbit anti-

ovalbumin serum was purchased from ICN (Costa Mesa, CA). Rabbit anti-Hsp105 was purchased from Santa Cruz biotech (Santa Cruz, CA). Anti-Flag monoclonal antibody M2 was purchased from Sigma (St. Louis, MO). Anti-Xpress antibody was purchased from Invitrogen (Carlsbad, CA). Anti-TAP1 and anti-Tapasin antibodies were kindly provided by Dr. Peter Cresswell (Yale University, New Haven, CT) or purchased from Stressgen (Victoria, BC, Canada) or Calbiochem (San Diego, CA). Rabbit anti-calreticulin and anti-calnexin, and anti-HSP27 monoclonal antibody was purchased from Stressgen. Rabbit anti-ORP150 (187) was kindly provided by M. Matsumoto (Osaka University Medical School, Suita City, Japan). HLA-A heavy chain specific mAB HCA2 and HLA-B,C specific mAB antibody HC10 (188) were kindly provided by H. Ploegh (Harvard Medical School).

Proteins and peptides

Ovalbumin (grade VII) was purchased from Sigma. All peptides used in the experiments were synthesized by Corixa corporation (Seattle, WA). Lyophilized peptides were reconstituted in DMSO at a concentration of 10mM and stored at -20°C.

Reverse transcriptase PCR (RT-PCR)

COS-K^b cells (50%-75% confluent in 75 cm^2 plates) were trypsinized and washed with PBS three times. mRNA was extracted using with an RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A cDNA pool was generated from COS-K^b mRNA using SuperScript II RNase H- Reverse transcriptase kit (Invitrogen) according to the manufacturer's protocol.PCR using this cDNA as template was performed using Platinum Pfx DNA Polymerase kit (Invitrogen) according to the manufacturer's protocol.

Plasmid vectors

Both pTracer-CMV (Invitrogen) and pTre2 vector (Invitrogen) were kindly provided by Dr. E.J. Luna. pTracer-CMV2, pcDNA3.1, pcDNA3.1/His A, B, C were purchased from Invitrogen (Carlsbad, California), pTet-ON plasmid and pBI-EGFP were purchased from Clontech (Palo Alto, CA) .

Expression plasmids

pTracer-CMV-UBC9: pCR3.1-UBC9 (128) was kindly provided by Dr. Mu-En Lee (Harvard School of Public Health). To add a flag tag to the N-terminus of UBC9, UBC9 was amplified with: Forward Primer (with EcoRI site): 5'-CGGAATTCCATGTCGGGGATTGCCCTCAGC-3'; Reverse Primer (with KpnI site): 5'-GGGGTACCAATCCCTTCCTCGTCATG-3'. The PCR product was digested with EcoRI and KpnI, and ligated to pCMV2 vector, which had a FLAG tag (kindly provided by Vincent Chau, Pennsylvania State University)). Flag-UBC9 was then amplified with forward primer (with KpnI): 5'-GGGGTACCGTCAGAATTGATCTAC-3', Reverse Primer (with KpnI): 5'-GGGGTACCAATCCCTTCCTCGTCATG-3'. The PCR product was digested with KpnI and ligated to KpnI digested pTracer-CMV-Vector. The insert direction was confirmed by enzyme digestion and DNA sequencing.

Mutant UBC9, in which the active cysteine has been changed to serine, was made by site-directed point mutation approach based on the protocol described by LJ Zhao (189), in which a mismatched oligonucleotide is extended, incorporating the "mutation" into a strand

of DNA that can be cloned. For making UBC9OH (in which the active amino acid cysteine 93 of UBC9 was mutated to serine), the inside primers were 5'-CAGGATGGACAGGGACACTGTGCCAG-3' (primer 2) and 5'-TCTGGCACAGTGTCCCTGTCCATCCTG-3' (primer 3).

A plasmid containing SUMO-1 (131) was kindly provided by R.T. Hay (University of St Andrews, St Andrews, UK); Ube2G2 (190) was kindly provided by S.A. Rose (University of Leeds, UK); and ubiquitin (191) was provided by R.R. Kopito (Stanford University, Stanford, California),

All other genes were cloned from a COS-7 cDNA pool by PCR amplification. The following is a list of primers used for making wild type and mutant plasmids of each gene:

Table 1

Gene name	Primers	Sequence (5'→3')	Note
UBC9	Primer 1 Primer 4	CGGAATTCCATGTCGGGGATTGCCCTCAGC GGGGTACCAATCCCTTCCTCGTCATG	Underlined is EcoRI site KpnI
UBC9OH	Primer 2 Primer 3	CAGGATGGACAGGGACACTGTGCCAG TCTGGCACAGTGTCCCTGTCCATCCTG	For making mutant gene, primer 1 and 4 are used
pTracerHA-SUMO-1	Primer 1 Primer 4	AAACTGCAGTGTCTGACCAGGAGGCAAAACC CGCTCTAGACTAAACTGTTGAATGACC	Pst I Xba I
pTracerHA-SUMO-1 (mut)	Primer 4 (mut)	CGCTCTAGATCACGTTTGTTCCTGATAAACTTC	Xba I
pTracerHA-SUMO-2	Primer 1 Primer 4	GCGGAATTCCATGTCCGAGGAGAAGCCCAAGG TCATCGTGGTGAATGTCCTCG	EcoR I. Primer 4 has no cutting site. But there is a XbaI within the product, after the stop codon
pTracerHA-SUMO-2 (mut)	Primer 2 Primer 3	CTCCGGCACAGCTGCCGTCTGCTG CAGCAGACGGCAGCTGTGCCGGAG	Two GG active aa have been changed to AA
pTracerHA-SUMO-3	Primer 1 Primer 4	GCGGAATTCCATGGCCGACGAAAAGCCCAAGG GGCTCTAGATCAGTAGACACCTCCCGTCTG	EcoR I Xba I
pTracerHA-SUMO-3 (mut)	Primer 4 (mut)	GGCTCTAGATCAGTAGACAGCTGCCGTCTGCTG	Xba I
pTracer-CMV-Ube2G1	Primer 1 Primer 4	TCAGAATTCCATGACGGAGCTGCAGTCGGCAC ATGCGGTACCTCACTCAAAAGCAGTCTCTTGG	EcoRI KpnI
pTracer-CMV-Ube2G1OH	Primer 2 Primer 3	GAAGAATAGAAATGGACACATCACC GGTGATGTGTCCATTTCTATTCTTC	C > S
pTracer-CMV-	Primer 1	TAGAATTCCATGGCGGGGACCGCGCTC	EcoRI

Ube2G2	Primer 4	GGGGTACCTCACAGTCCCAGAGACTTCTGG	KpnI
pTracer-CMV-Ube2G2OH	Primer 2 Primer 3	GGAGGATGGAAATGGAGACTCTCCCATCAG GGGAGAGTCTCCATTTCCATCCTCCAC	
pTracer-CMV-Ubiquitin	Primer F Primer R	GCAGGAATTCATTATGCAGATC GCGCGAATTCTCAACCACCTCTTAGTCTTAAG	EcoRI EcoRI
pTracer-HA-HSP27	Primer F Primer R	TAGCGGAATTCCAGCATGACCGAGCGCCGCGTC TAGGCTCTAGACGGGCTAAGGCTTTACTTGGC	EcoRI XbaI

Other plasmids were constructed using standard cloning techniques (192). All the plasmid DNA sequences were confirmed by sequencing (Nuclear Acids Facility, University of Massachusetts Medical School, Worcester, MA).

Table 2

Plasmid Name	Description
pTracer-CMV	Cloning vector expressing GFP
pTracer-CMV-UBC9	pTracer-CMV expressing wild type UBC9 (Flag tagged)
pTracer-CMV-UBC9OH	pTracer-CMV expressing mutant UBC9 (C93S) (Flag tagged)
pTracer-CD16-Ova	pTracer-CMV expressing Ovalbumin targeted to ER by CD16 leader signal sequence
pTracer-N50-Ova	pTracer-CMV expressing cytosolic OVA with the N-terminal 50 amino acids deleted
pTracer-ES-Minigene	pTracer-CMV expressing SIINFEKL targeted to ER by Ad5 E3gp19K leader signal sequence
pTracer-Minigene	pTracer-CMV expressing MSIINFEKL
pTracer-Ubc2G1	pTracer-CMV expressing wild type Ube2G1 (Flag tagged)
pTracer-Ubc2G1OH	pTracer-CMV expressing mutant Ube2G1 (Cys -> Ser) (Flag tagged)
pTracer-Ubc2G2	pTracer-CMV expressing wild type Ube2G2 (Flag tagged)
pTracer-Ubc2G2OH	pTracer-CMV expressing mutant Ube2G2 (Cys -> Ser) (Flag tagged)
pTracer-HSP27	pTracer-CMV expressing HSP27
pTracer-TOP	pTracer-CMV expressing TOP
pTracer-Ubc14	pTracer-CMV expressing wild type Ubc14 (Flag tagged)
pTracer-Ubc14OH	pTracer-CMV expressing mutant Ubc14 (Cys -> Ser) (Flag tagged)
pTracer-Ubc15	pTracer-CMV expressing wild type Ubc15 (Flag tagged)
pTracer-Ubc15OH	pTracer-CMV expressing mutant Ubc15 (Cys -> Ser) (Flag tagged)
pTracer-CMV-HA vector	Cloning vector expressing GFP, with HA tag at the N-term.
pTracer- PSA	pTracer-CMV- expressing Puromycin sensitive aminopeptidase
pTracer-HA-HuBH	pTracer-CMV-HA expressing Human Bleomycin Hydrolase
pTracer-HA-SUMO-1	pTracer-CMV-HA expressing wild type SUMO-1
pTracer-HA-SUMO-1(mt)	pTracer-CMV-HA expressing mutant SUMO-1 (C-term GG have been deleted)
pTracer-HA-SUMO-2	pTracer-CMV-HA expressing wild type SUMO-2
pTracer-HA-SUMO-2(mt)	pTracer-CMV-HA expressing mutant SUMO-2 (C-term GG have been changed to AA)
pTracer-HA-SUMO-3	pTracer-CMV-HA expressing wild type SUMO-3
pTracer-HA-SUMO-3(mt)	pTracer-CMV-HA expressing mutant SUMO-3 (C-term GG have been deleted)
pcDNA3.1	Expression cloning vector does not express GFP
pcDNA3.1-CD16-OVA	pcDNA3.1 expressing Ovalbumin targeted to ER by CD16 leader signal sequence
pcDNA3.1-N50-OVA	pcDNA3.1 expressing cytosolic OVA with N-terminal 50 amino acids deletion
pcDNA3.1-ES-minigen	pcDNA3.1 expressing SIINFEKL targeted to ER by CD16 leader signal sequence
pcDNA3.1-Minigene	pcDNA3.1 expressing MSIINFEKL
pcDNA3.1-N25-Mini	pcDNA3.1 expressing SIINFEKL with 25 extra flanking amino acids at the N-terminal
pcDNA3.1-N15-Mini	pcDNA3.1 expressing SIINFEKL with 15 extra flanking amino acids at the N-terminal
pcDNA3.1-N5-Mini	pcDNA3.1 expressing SIINFEKL with 5 extra flanking amino acids at the N-terminal
pcDNA3.1-C5-Mini	pcDNA3.1 expressing SIINFEKL with 5 extra flanking amino acids at the C-terminal
pcDNA3.1-C15-Mini	pcDNA3.1 expressing SIINFEKL with 15 extra flanking amino acids at the C-terminal
pcDNA3.1/His A, B, C	Expression cloning vector for generating His tagged protein, and it has X-press epitope
pcDNA3.1/His-SUMO-2	pcDNA3.1/His expressing wild type SUMO-2
pcDNA3.1/His-SUMO-2(mt)	pcDNA3.1/His expressing mutant SUMO-2 (C-terminal GG has been changed to AA)

pcDNA3.1/His-HuBH	pcDNA3.1/His expressing wild type HuBH
pcDNA3.1/His-HuBH(mt)	pcDNA3.1/His expressing mutant HuBH (C-terminal two amino acids deleted, and point mutation of C73A)
pTet-ON	A plasmid expressing the reverse tet-responsive transcriptional activator (rtTA)
pTet-ON-UBC9	pTet-ON in which UBC9 has been inserted and driven by CMV promoter
pTre2	pTRE2 is an tetracycline inducible plasmid to express a gene of interest in the presence of pTet-On
pTre2-CD16-OVA	pTre2 expressing Ovalbumin targeted to ER by CD16 leader signal sequence
pTre2-N50-OVA	pTre2 expressing N50-Ovalbumin (see above)
pTre2-ES-Minigene	pTre2 expressing SIINFEKL targeted to ER by Ad5 E3 gp19K leader signal sequence
pTre2-Minigene	pTre2 expressing MSIINFEKL
pBI-EGFP	pBI-EGFP is a tetracycline inducible plasmid to co-express a gene of interest and enhanced green fluorescent protein (EGFP) from a bidirectional tetracycline-responsive promoter (P_{bi-1})
pBI-EGFP-CD16-OVA	pBI-EGFP expressing Ovalbumin targeted to ER by CD16 leader signal sequence
pBI-EGFP-N50-OVA	pBI-EGFP expressing N50-Ovalbumin (see above)
pBI-EGFP-ES-Minigene	pBI-EGFP expressing SIINFEKL targeted to ER by Ad5 E3 gp19k leader signal sequence
pBI-EGFP-Minigene	pBI-EGFP expressing MSIINFEKL
3.2.1	pCDL-SRα296 plasmid expressing CD16-OVA-LAMP.1, which targets the protein to lysosome

Transfection:

Cells were transfected with Fugene 6 (Roche Applied Science, Indianapolis, IN) according to the manufacture's instructions. Briefly, the day before transfection, cells were plated on 6-well plates or 6-cm diameter tissue culture dishes at $3x10^5$/well (for 6-well plates) or $7x10^5$/dish (for 6-cm dishes). 24 hour later, cells (at confluency of 50% - 70%) were transfected with plasmid(s). The ratio of Fugene6: DNA was 3 μl:1 μg. After transfection, the cells were incubated at 37°C, 5% CO_2 for 20-48 hours. For co-transfection, plasmids were mixed first, and then mixed with FuGene 6.

Selecting low H-2K^b expressing COS-K^b cells with MACS

Magnetic bead activated cell sorting (MACS) was done according to the manufacturer's protocol (Miltenyi Biotech, Auburn, CA). Briefly, COS-K^b cells were trypsinized and washed 2 times with complete RPMI1640 medium, re-suspended in H-2K^b-specific monoclonal antibody Y3 supernatant (180), and incubated on ice for 15-20 minutes. After two washes with cold PBS buffer supplemented with 5% FBS (PBS/FBS), the cells were re-

suspended with goat anti-mouse IgG magnetic beads (Miltenyi Biotech, Auburn, CA) (1:100 – 1:150 diluted in PBS/FBS), and incubated on ice for a further 15 minutes. Negative selection was done with MACS separation column (LS or CS). The flow through (low H-2K^b-expressing) cells were collected and grown in G418-free complete RPMI1640 medium.

Generating ovalbumin stable transfectants

To generate stable transfectants, COS-K^b cells were transfected with pcDNA3.1/Hygro-CD-16-OVA, pcDNA3.1/Hygro-N50-OVA, or pcDNA3.1/Hygro-minigene. Twenty-four hours after transfection, the cells were trypsinized and seeded in medium containing 300 μg/ml hygromycin in new dishes. Two days later, the medium was changed, the cells were grown in the presence of hygromycin for 3 more days, and then limit-diluted into 96-well plates. Single colonies were selected and expanded in 24-well plates and then in 6-well plates. Cells staining with 26.D1.16 (anti-SIINFEKL+H-2K^b) were used for studying UBC9 function in MHC class I antigen presentation.

Antibody staining and FACS analysis

Cells were trypsinized and washed with complete RPMI1640 medium twice, re-suspended in 100 μl antibody supernantant and incubated on ice for 45 minutes. After two washes with cold PBS buffer supplemented with 5% FBS, the cells were re-suspended with Cy5-conjugated goat anti-mouse IgG antibodies or PE-conjugated donkey anti mouse IgG antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) (1:100 – 1:150 diluted in PBS/FBS), and incubated on ice for a further 45 minutes. The cells were then washed

with cold PBS 2 times and examined on a FACSCalibur apparatus (Becton Dickinson, San Jose, CA) using CELLQUEST software (Becton- Dickinson) and analyzed with Flowjo software (Tree Star, Inc. San Carlos, CA). When cells were transfected with GFP-expressing plasmids, such as pTracer-CMV, analysis was restricted to GFP-positive cells.

Inducible ovalbumin expression

Cos-K^b cells were co-transfected with pTet-ON-Vector or pTet-ON-UBC9, and pBI-EGFP-CD16-OVA, pBI-EGFP-N50-Ova, pBI-EGFP-ES-Minigene or pBI-EGFP-Minigene. Twenty-four hours after transfection, the cells were trypsinized and washed with RPMI1640 (serum-free) 2 times. To remove any preexisting peptide-MHC complexes, cells were acid-washed with 1 ml acid washing buffer (1:1 mixture of 0.163M citric acid and 0.32M NaH_2PO_4) for less than 1 minute. The acid buffer was neutralized with 10ml cold Na_2HPO_4 buffer (1 M, pH7.5). The cells were washed with cold Opti-MEM 2 more times and grown in RPMI1640 complete medium containing doxycycline (20 μg/ml) at 37°C and analyzed for the presence of peptide-MHC complexes by flow cytometry at different time points.

Protein or peptide loading experiments with hypertonic solution

Cos-K^b cells were transfected with pTracer-CMV-vector or expression plasmids. Twenty eight hours after transfection, the cells were trypsinized, washed 3 times with serum-free RPMI1640 medium and transferred to a 14 ml tube. Anigen was loaded into the cytosol by hypertonic lysis of pinosomes. The cells were re-suspended in 200μl loading buffer (described in appendix) containing ovalbumin (80 mg/ml) or peptides (0.2mg/ml for N5-SIINFEKL, 0.1mg/ml for SIINFEKL, 0.5mg/ml for SIINFEKLT). After incubating in a

37°C water-bath for 10 minutes, 2.5ml pre-warmed hypotonic solution (described in appendix) was added, mixed, and the cells were incubated at 37°C for 3 minutes. The cells were spun down and washed with ice-cold SF-Opti-Mem (see Appendix I) medium four times to remove excess antigen. For peptide loading, after one wash with ice-cold SF-Opti-Mem medium, the cells were acid-washed once and then washed with ice-cold SF-Opti-Mem medium twice (193, 194). The cells were re-suspended in complete RPMI1640 medium and grown at 37°C in 5% incubator. At various time points, cells were analyzed by flow cytometry.

Peptide protection experiments: Antigen presentation assay

Cos-7 cells were plated in 15-cm diameter dishes ($3x10^6$ cells/dish). Twenty-four hours later, the cells were transfected with pTracer-CMV-vector or pTracer-CMV-UBC9 plasmid (25μg DNA and 75μl Fugene6 per dish). Twenty-eight hours after transfection, the cells were loaded with SIINFEKL peptide (0.5mg/ml) with hypertonic solution (appendix), and washed 3 times with serum-free RPMI1640 medium. The cells were re-suspended in complete RPMI1640 medium, and grown at 37 °C. At various times cells were lysed with 10% TCA or TFA and boiled for 15 minutes, and then incubated on ice for 10 minutes. The cell lysates were neutralized with NaOH, centrifuged at 14,000 rpm for 10 min, and the supernatant (peptide extract) was transferred to another eppendorf tube. This peptide extract was then assayed for the presence of SIINFEKL with a T cell hybridoma assay.

The peptide extract was titered in 2-fold dilutions across a 96-well plate (Corning-Costar, Acton, MA) (50μl/well), and fixed E36-Kb and RF33.70 cells were added at

100µl/well (1 x 10^5 cells/well) and incubated 18-24 hrs at 37°C in 5% CO_2. To fix theE36-Kb cells, they were washed 3 times with PBS and fixed with 1% paraformaldehyde in PBS for 15 minutes at room temperature. The cells were washed with two volumes of RPMI 1640 three times. RF33.70 cells (specific for SIINFEKL complexed with H-$2K^b$) were washed twice with RPMI1640/FCS medium and resuspended with the fixed E36-Kb cells in hybridoma culture medium (HCM) at 1 x 10^6 cells/ml each. One hundred µl supernatant was frozen at -20°C overnight and used in an IL-2 assay.

IL-2 assays were performed as described in (47). Briefly, CTLL-2 cells were washed three times with RPMI1640+5% FCS, resuspended at 2x10^5/ml in HCM with an equal volume of nutritional cocktail (50 ml of RPMI supplemented with 1 ml of dextrose [500 mg/ml stock in RPMI], 2 ml of essential amino acids, 1 ml of non-essential amino acids, 1 ml of L-glutamine, 1 ml of penicillin-streptomycin-fungizone, and 50 ml of FBS). This cell suspension was added to wells at 5x10^3 cells/well. After 18-22 hours at 37°C, 5% CO_2 the cells were pulsed with ^{3}H-thymidine media (1 ml 1mci/ml ^{3}H thymidine in 24 ml HCM). After 5 or 6 hours supernatant was harvested and incorporated ^{3}H thymidine counts were measured in a Microbeta liquid scintillation and Luminescence counter (PerkinElmer Wallac Inc, Gaithersburg, MD)

Peptide protection experiments – HPLC experiments

COS-7 cells were grown to 50-70% confluency in 150 cm^2 culture plates and transfected with pTracer-CMV-Vector or pTracer-CMV-UBC9. Twenty-eight hours after transfection, the cells were placed on ice and culture medium was discarded. Cells were

washed three times with ice-cold PBS, pH 7.4, and remaining PBS was removed. Cells were covered with 1.5 ml of homogenization buffer (50 mM Hepes, pH 7.4 adjusted with KOH, 2 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT), and scraped off the plates into the buffer with a cell lifter. This cell suspension was then used to harvest cells in the second plate, and so on until cells in all plates were harvested in 1.5 ml of the homogenization buffer.

Cell suspensions were transferred to a 7-10 ml (or smaller) glass douncing homogenizer on ice. After douncing about 40 times, the homogenates were transfered to 1.5 ml Eppendorf tubes and centrifuged at 14,000 rpm for 10 min at 4°C in Eppendorf centrifuge to remove nuclei, mitochondria, lysosomes and plasma membranes. The supernatants from the first spin were additionally centrifuged at 100,000xg for 2 hours. Protein concentration in 100,000xg supernatants was determined by the Bradford assay (Pierce, Rockford, IL). Samples were aliquoted into 100 µl aliquots / 0.5 ml tube. Aliquots were snap-frozen in liquid nitrogen, and kept at –80°C.

Five nmol of ESIINFEKL peptide was incubated at 37 °C for various time intervals with 10 µg extracts from COS7 cells transfected with pTracer-CMV vector or pTracer-CMV-UBC9, in 100 µl of 50 mM Tris-HCl, pH 8.5, 5 mM $MgCl_2$, 20 µM MG132. The reaction was terminated by the addition of 100 µl of 20% trichloroacetic acid followed by a 15-min incubation on ice and removal of the precipitated protein by centrifugation for 15 min at 20,000 g. The peptide-containing supernatant was subjected to reverse-phase HPLC on a 4.6 x 250-mm Vydac 5-µm C18 column (Vydac, Hesperia, CA) in 0.06% trifluoroacetic acid with a flow rate of 1 ml/min. Elution was performed with a 30-min linear

gradient from 5 to 60% acetonitrile. The amounts of eluting peptides were calculated by integration of peptide peaks on chromatograms and are given in arbitrary units (195).

Pulse-chase Immunoprecipitation

Cos-Kb cells were plated on 75 cm^2 plates and transfected with expression plasmids. Twenty-eight hours after transfection, the transfected cells were trypsinized and washed for 2 times with labeling medium (methionine and cysteine-free RPMI1640 [Sigma] supplemented with L-glutamine and 10% dialyzed FCS). Cells were resuspended with 2 ml of labeling medium, and incubated at 37 °C in 5% CO_2 for 1 hour. Twenty-five µl (~250µCi) of [^{35}S] methionine/cysteine (EasyTag, PerkinElmer Life Sciences Inc., Boston, MA) was added per well and incubated for 5-20 minutes (Pulse). The cells were washed with non-radioactive complete medium 2 times, and resuspended in 3.5 ml chasing medium (RPMI1640 supplemented with 500 µg/ml cysteine-HCl, 100µg/ml methionine, sterile filtered), and grown at 37°C in 5% CO_2. Cell suspensions were taken at different time points and washed 2 times with PBS. Cells were lysed with lysis buffer (1% NP40 in 150mM NaCl, 50mM Tris pH 8.0, 1mM EDTA) supplemented with complete protease inhibitors (EDTA free) (Roche) on ice for at least 15 minutes. After centrifugation at 14,000 rpm for 10 minutes, the supernatants were collected, and subjected to immunoprecipitation.

Protein A-agarose beads [Repligen, Waltham, MA] were incubated with irrelevant antibody or with normal rabbit serum in lysis buffer for 1 hour at 4 °C. The beads were washed briefly, resuspended with cell lysates, and incubated for 1 hour at 4 °C to preclear

the lysates. Protein A beads pre-incubated with specific antibody were added to the cleared lysates and incubated for 2 hours at 4 °C. The beads were washed with lysis buffer 3 times, resuspended with Laemmli's sample buffer, and heated to 100 °C for 10 minutes. Samples were separated by SDS-PAGE on 12% acrylamide, treated with Autofluor (National Diagnostics, Atlanta, Georgia) for 30 minutes, dried, and exposed to X-ray film for appropriate periods at –80 °C.

Protease inhibition experiments

Cos-K^b cells were transfected with pTracer-CMV-vector or pTracer-CMV-UBC9. Twenty-eight hours after transfection, the cells were trypisinized, washed 3 times with serum-free RPMI1640 medium and grown in medium containing protease inhibitors for 1 hour. The inhibitors were: proteasome inhibitor β-lactone; inhibitor of thiol proteases (such as Cathepsin B and BH) E64; calpain and calpain II inhibitor E-64C; serine protease inhibitor AAF-CMK; leucine aminopeptidase inhibitor bestatin; leupeptin, which inhibits chymotrypsin, plasmin, papain and cathepsin B; chymostatin, which inhibitis chymotrypsin, papain; and cathepsins A, B, and D inhibitor chymostatin. All inhibitors were used at 100μM except for leupeptin, which was used at 1mM. The cells were then hypotonically loaded with ovalbumin, incubated in inhibitor-containing medium, and analyzed by flow cytometry.

Fluorescent immunocytochemistry.

The day before transfection, Cos-K^b cells were plated in 24 well plates on a cover glass. Cells were transfected with pTracer-CMV-Vector or pTracer-CMV-UBC9 (Flag tagged),

pCMV2-UBC9 (wild type) or pcDNA3. Twenty-eight hours after transfection, the cells were fixed with dry-ice cold acetone/methanol (1:1) for 1 min and air-dried for 10 minutes, rehydrated in PBS for at least 2 minutes, and blocked with blocking buffer (2% normal goat serum in PBS), for 1 hour at room temperature. Wells were drained completely. Cells were stained with 250μl primary antibody (1:100 diluted in blocking buffer) for 45 minutes, and then washed for 3 times with PBS, 10 minutes/time and stained with 250μl secondary antibodies (rhodamine-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-mouse IgG, 1:100 diluted in blocking buffer) for 45 minutes and washed for 3 times with PBS, 10 minutes/time. Cells were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate, 1 μg/ml) for 10-20 minutes, and washed twice with PBS, 10 minutes/time. Cover glasses were mounted onto slides with Fluoromount-G (Southernbiotech Inc. Birmingham, AL), dried overnight at room temperature and stored at -20°C until fluorescence microscope examination.

IFNγ and poly I:C treatment of COS cells and Dendritic cells

To know whether IFNγ and IFNα have effect on UBC9 expression, COS-Kb cells, bone marrow-derived primary mouse dendritic cells, monocyte-derived human dendritic cells, or DC2.4 cells were grown in 6-well plates with FNγ (250u/ml/well) (Biogen, Cambridge IFNγ, MA) or polyI:C (Sigma) (25 μg/ml, 5μg/ml, 2.5 μg/ml, and 1μg/ml). The cells were cells trypsinized after 1 day, 2 days, and 3 days, and analyzed on western blots as described below.

Polyacrylamide gel electrophoresis and immunoblotting

After cell lysis as described above 1/3 volume of 3X loading buffer (NEB, Beverly, MA) and DTT (NEB) was added to the samples, and they were then boiled for 5-10 minutes. Samples were subjected to SDS-PAGE. Proteins were then transferred from the gel to 0.22μm nitrocellulose membrane PROTRAN (Schleicher and Schuell, West Chester, PA) at 67V for 2.5 hours. The membranes were then removed from the transfer apparatus and placed into blocking solution (5% fat-free milk in PBS with 0.2% Tween 20 [PBST]) overnight at room temperature. The membranes were then incubated with primary antibody diluted in blocking buffer as suggested in the product description sheet, and incubated with agitation for 1 hour at room temperature, washed for 30-60 minutes with agitation in PBST buffer, changing the wash buffer every 10-15 minutes, and incubated with HRP-conjugated secondary antibody diluted in 5% non-fat dry milk in wash buffer (sodium azide-free) for 1 hour. The membranes were washed again for 30 minutes to 1 hour for monoclonal antibodies, and at least 2 hours for rabbit polyclonal antibodies. The blots were developed using SuperSignal West Pico Substrate (Pierce, Rockford, IL) for 5 minutes. The membranes were dried by touching against paper towers, wrapped with plastic wrap, and exposed to X-ray film (Eastman Kodak Company, Rochester, New York).

Small interfering RNA (siRNA) treatment

All siRNA used in the experiments was synthesized by Dharmacon Research, Inc (Lafayette, CO), and was annealed exactly as described (196). Double stranded RNA specific for human UBC9 mRNA are 5'-AUGUAAAUUCGAACCACCAdTdT-3' (sense); and 5'-UGGUGGUUCGAAUUUACAUdTdT-3' (complement). Double stranded RNA

specific for human HSP27 mRNA are 5'-GUUUCCUCCUCCCUGUCCCdTdT-3' (sense) and 5'-GGGACAGGGAGGAGGAAACdTdT-3' (complement).

Cos-K^b cells were grown in 6-well plates at $1.5x10^5$/well. Twenty four hours later the cells were transfected with siRNA, using Transit TKO (Mirus, Madison, WI), following the protocol provided by the manufacturer. Briefly, Transit-TKO reagent (3 μl) was added dropwise into 50 μl Opti-MEM, mixed thoroughly and incubated at room temperature for 5 to 20 minutes. Five μl of 20 μM siRNA was added to the diluted Transit-TKO reagent, mixed well and incubated at room temperature for 5 to 20 minutes. The Transit-TKO/siRNA mixture was added dropwise into the cells, while swirling the plates. Cells were incubated for 24, 48, and 72, and 96 hours before analysis.

CHAPTER III. RESULTS

3.1. UBC9 overexpression increases surface H-2K^b-SIINFEKL levels

To evaluate the effect of UBC9 on antigen presentation, I co-transfected COS-K^b cells with pTracer-CMV-UBC9, or pTracer-CMV vector, and pcDNA3.1-CD16-OVA (which targets the protein to ER) (see Table 2), and after 24 hours measured the levels of H-2K^b-SIINFEKL complexes at the cell surface with the H-2K^b-SIINFEKL complex-specific monoclonal antibody 25.D1.16 (178). The pTracer-CMV vector co-expresses GFP, so transfected (GFP+) cell populations were gated and analyzed in flow cytometry. pTracer-CMV-UBC9 and pTracer-CMV-UBC9OH plasmids expressed high levels of UBC9 protein in COS-K^b cells (Fig 1A). Compared to vector-transfected cells, those expressing UBC9 showed markedly increased expression of H-2K^b-SIINFEKL complexes (Fig 1B). Interestingly, the mutant UBC9OH, in which the catalytic cysteine 93 has been mutated to serine and which should not be able to conjugate ubiquitin or sumo to substrates (157) , had the same effect as its wild-type counterpart in increasing H-2K^b-SIINFEKL level (Fig 1C). But this effect is not due to UBC9 increasing protein synthesis, since both wild type and mutant UBC9 did not increase ovalbumin and HLA-A3 protein protein levels (Fig 1D, E). These observations led us to study the role of UBC9 in MHC class I antigen presentation.

3.1.1. UBC9 overexpression increases surface H-2K^b expression

The amount of class I molecules expressed on the cell surface is strongly influenced by the supply of antigenic peptides in cells. To know whether UBC9 overexpression affected MHC class I antigen presentation generally or was limited to SIINFEKL or only a few other

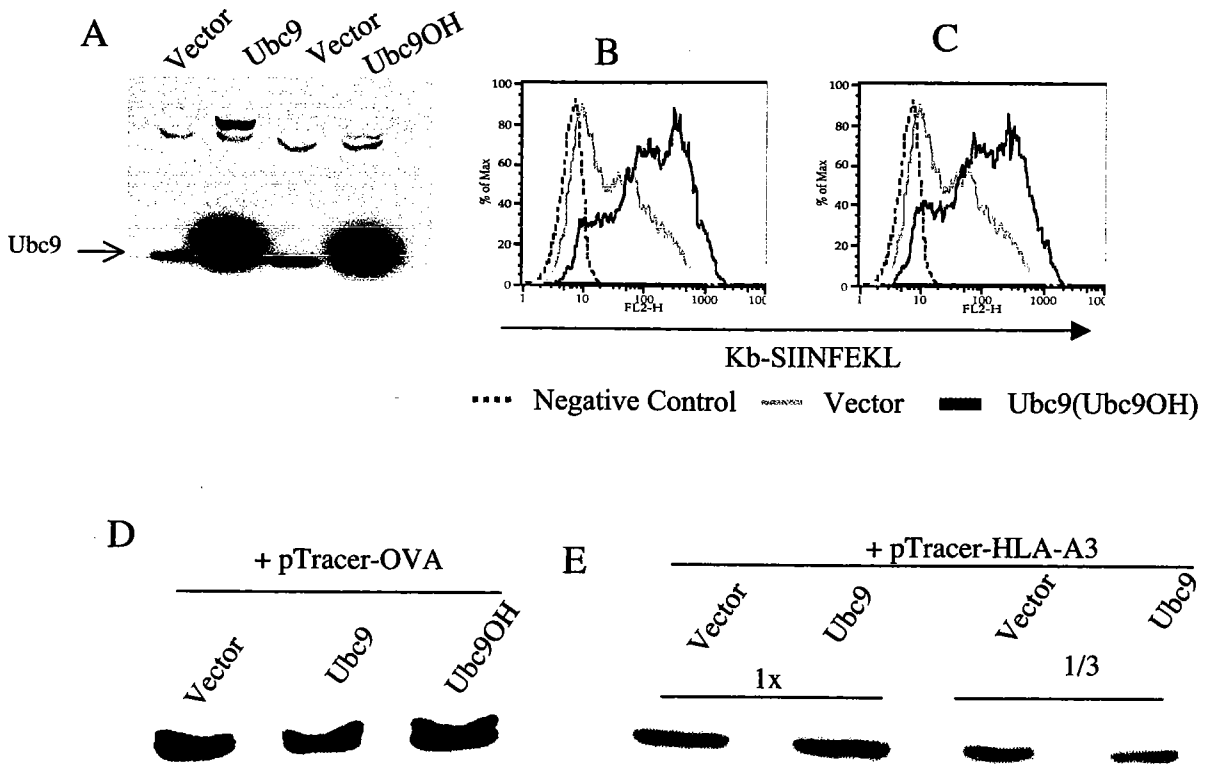


Fig 1. COS-Kb cells transfected with pTracer-CMV-UBC9 and pTracer-CMV-UBC9OH express high levels of UBC9 and increase surfaceH-2K^b-SIINFEKL levels without increasing protein synthesis. A. COS-K^b cells were transfected with vector, pTracer-CMV-FLAG-UBC9, or pTracer-CMV-FLAG-UBC9. Twenty-four hours after transfection, the cells were lysed and a western blot was performed with anti-UBC9 antibody. B and C. COS-Kb cells were co-transfected with ovalbumin plasmid and pTracer-CMV-Vector, or pTracer-CMV-Ubc9 (B) or pTracer-CMV-Ubc9OH (C). Twenty-four hours after transfection, the cells were stained with H-2Kb-SIINFEKL specific antibody 25..D1.16 and GFP+ populations were analyzed by flow cytometry. E and F: COS-K^b cells were co-transfected with pTracer-OVA and control vector, Ubc9, or Ubc9OH (E); or COS-K^b cells were co-transfected with pTracer-HLA-A3 and control vector or Ubc9 (F). Western blot was done 24 hours after transfection. Membranes were probed with anti-OVA and anti-A3 antibody (HCA2) respectively.

epitopes, we transfected Cos-K^b cells with pTracer-CMV-UBC9 and pTracer-CMV-UBC9OH and measured the effects on overall MHC class I expression on the cell surface. We found that UBC9 overexpression has very little effect on endogenous MHC class I antigen presentation (Fig 2A), and only slightly increased H-$2K^b$ levels (Fig 2B), though the effect was not as dramatic as the increase in K^b-SIINFEKL levels described above (section 1). Since these COS-K^b cells express very high levels of H-$2K^b$, it seemed possible that its expression was already at a maximum and could not be further increased by enhancing peptide supply. If so, the effect of UBC9 on MHC class I antigen presentation (such as peptide supply for H-$2K^b$) might be difficult to detect.

To circumvent this potential limitation, we used the magnetic cell sorting (MACS) technique to select COS-Kb clones that expressed low levels of H-$2K^b$, followed by limiting-dilution subcloning. We selected a clone that expressed low but easily-detectable levels of H-$2K^b$ on the cell surface. These cells were transfected with pTracer-CMV vector, pTracer-CMV-UBC9 or pTracer-CMV-UBC9OH. Twenty-four hours after transfection, the cells were stained with an anti-Kb antibody (Y3). In these H-$2K^b$-low cells, UBC9 (Figure 3A) and UBC9OH (Figure 3B) overexpression strongly increased H-$2K^b$ level on cell surface, suggesting that UBC9 has a general enhancing effect on MHC class I antigen presentation.

3.1.2. UBC9 increases H-$2K^b$-SIINFEKL expression on cells transfected with all forms of SIINFEKL precursors

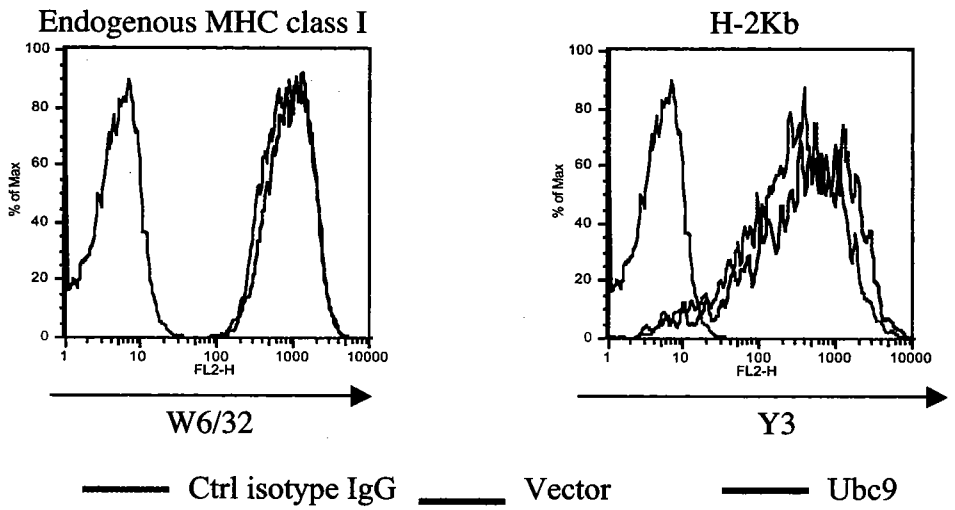


Fig 2. UBC9 overexpression has little effect on surface endogenous MHC class I levels. COS-K^b cells were transfected with UBC9 or control vector. Twenty-eight hours after transfection, the cells were stained with HLA-A, B, C specific antibody W6/32 or H-$2K^b$ specific antibody Y3 and GFP positive cells were analyzed by flow cytometry.

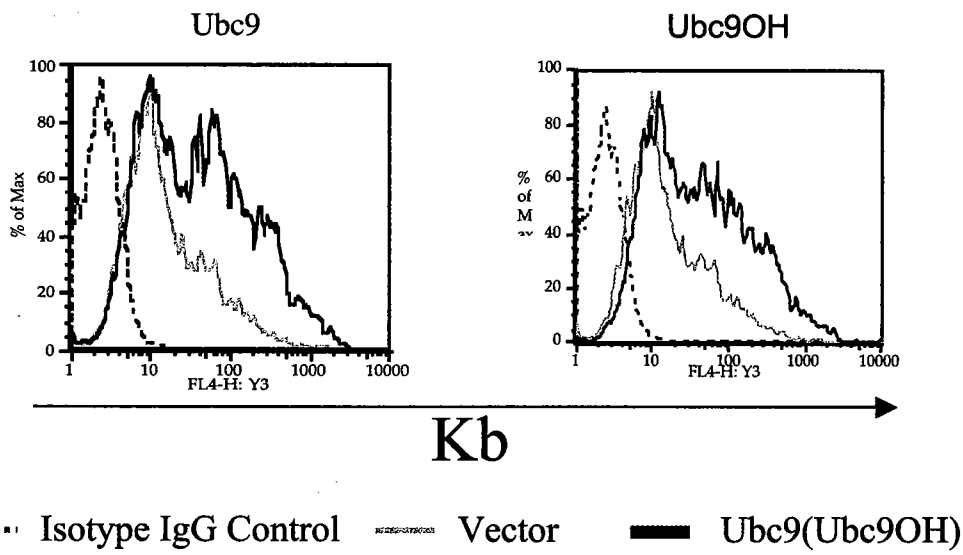


Fig 3: Ubc9 and mutant Ubc9 over-expression globally increases MHC class I antigen presentation. Low H-2Kb-expressing COS-Kb cells were transfected with pTracer-CMV vector, pTracer-CMV-Ubc9, or pTracer-CMV-Ubc9OH. Thirty-six hours after transfection, the cells were stained with H-2Kb-specific antibody Y3 and GFP+ cell populations were analyzed by flow cytometry.

MHC class I antigen processing and presentation follows a multi-step pathway involving gene transcription, protein synthesis, antigen degradation, peptide trimming, peptide transport, MHC complex assembly, peptide binding, and transport of complexes to the cell surface as MHC class I-peptide complexes. My observations above showed that UBC9 overexpression up-regulated MHC class I antigen presentation. To identify at which step(s) UBC9 affects MHC class I antigen presentation, I used ovalbumin as a model protein; different constructs were used to test different steps in the pathway.

Because UBC9 has been implicated in altering the subcellular localization of proteins, I initially tested whether UBC9 overexpression differentially affected ovalbumin targeted to different regions of the cell. Cos-K^b cells were co-transfected with UBC9 and 3.2.1 (pCDL-SRα296-CD16-OVA-LAMP.1) (which encodes endosome/lysosome-targeted ovalbumin), pcDNA3.1-CD16-OVA (which encodes ER lumen-targeted ovalbumin), or pcDNA3.1-N50-OVA (which targets ovalbumin to the cytosol). We found that UBC9 overexpression increased H-2K^b-SIINFEKL levels on Cos-K^b cells transfected with all these forms of ovalbumin (Figure 4A-D). The largest increases were found on cells transfected with ER-targeted and endosome-targeted ovalbumin (Figure 4A and 4B). UBC9 overexpression only moderately increased H-2K^b-SIINFEKL levels on cells transfected with cytosolic ovalbumin (N50-OVA) (Figure 4c). This initially suggested that UBC9 might enhance degradation of ER proteins specifically, for example by increasing retrograde translocation of the protein into the cytosol. However, cytosolic ovalbumin is very unstable (see below), which means that it is quickly degraded and may provide more peptides to MHC class I molecules. Since transient transfection leads to very high levels of protein expression in

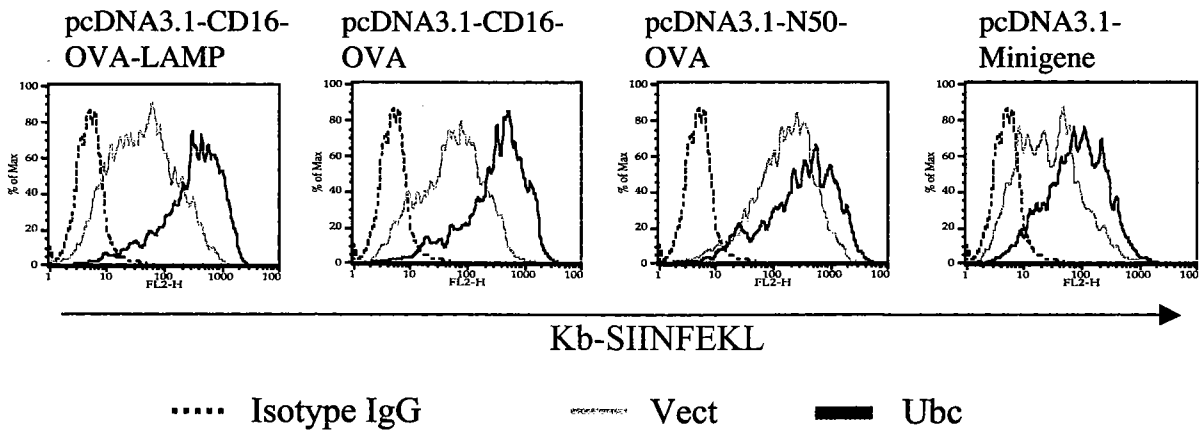


Fig 4: Ubc9 over-expression increases Kb-SIINFEKL level on cells transfected with many forms of ovalbumin. COS-Kb cells were co-transfected with pTracer-CMV-Vector or pTracer-CMV-Ubc9, and with pcDNA3.1-CD16-OVA-LAMP.1 (trgeted to the lysosome), pcDNA3.1-CD16-OVA (ER-targeted), pcDNA3.1-N50-OVA (cytosolic), or pcDNA3.1-Minigene (MSIINFEKL). Twenty-four hours after transfection, the cells were stained with H-2Kb-SIINFEK- specific antibody 25.D1.1 and GFP+ cell populations were analyzed by flow cytometry.

these COS cells, an alternate explanation was that expression of cytoplasmic ovalbumin leads to saturation of H-2K^b-SIINFEKL levels. This may obscure any enhancing effect of UBC9. As well, in transient transfections a potential complicating factor is interference between the co-transfected ovalbumin-expressing and UBC9-expressing plasmids. Both of these concerns could be at least partially addressed with stable transfectants, since stable constructs tend to express lower levels of protein and since I would be able to transiently transfect the cells with just a single plasmid, which would eliminate the potential interference of co-transfection.

3.1.3. UBC9 effects on stable ovalbumin transfectants

I generated several stable transfectants expressing ER-targeted ovalbumin and cytosol-targeted ovalbumin (Fig 5A). I then transiently transfected these stable transfectants with pTracer-CMV vector, pTracer-CMV-UBC9, or pTracer-CMV-UBC9OH. Twenty-six hours after transfection, the cells were analyzed by flow cytometry, and GFP-positive cell populations were analyzed (Fig 5B). In contrast to the transiently transfected cells, in the stable transfectants UBC9 overexpression markedly increased H-2K^b-SIINFEKL levels not only on ER-targeted ovalbumin (CD16-Ova), but also on cytosolic ovalbumin (N50-Ova) transfected cells. Therefore UBC9 overexpression increases H-2K^b-SIINFEKL level from ovalbumin regardless of its subcellular location.

3.1.4. UBC9 does not increase H-2K^b-SIINFEKL levels by enhancing the synthesis of ovalbumin

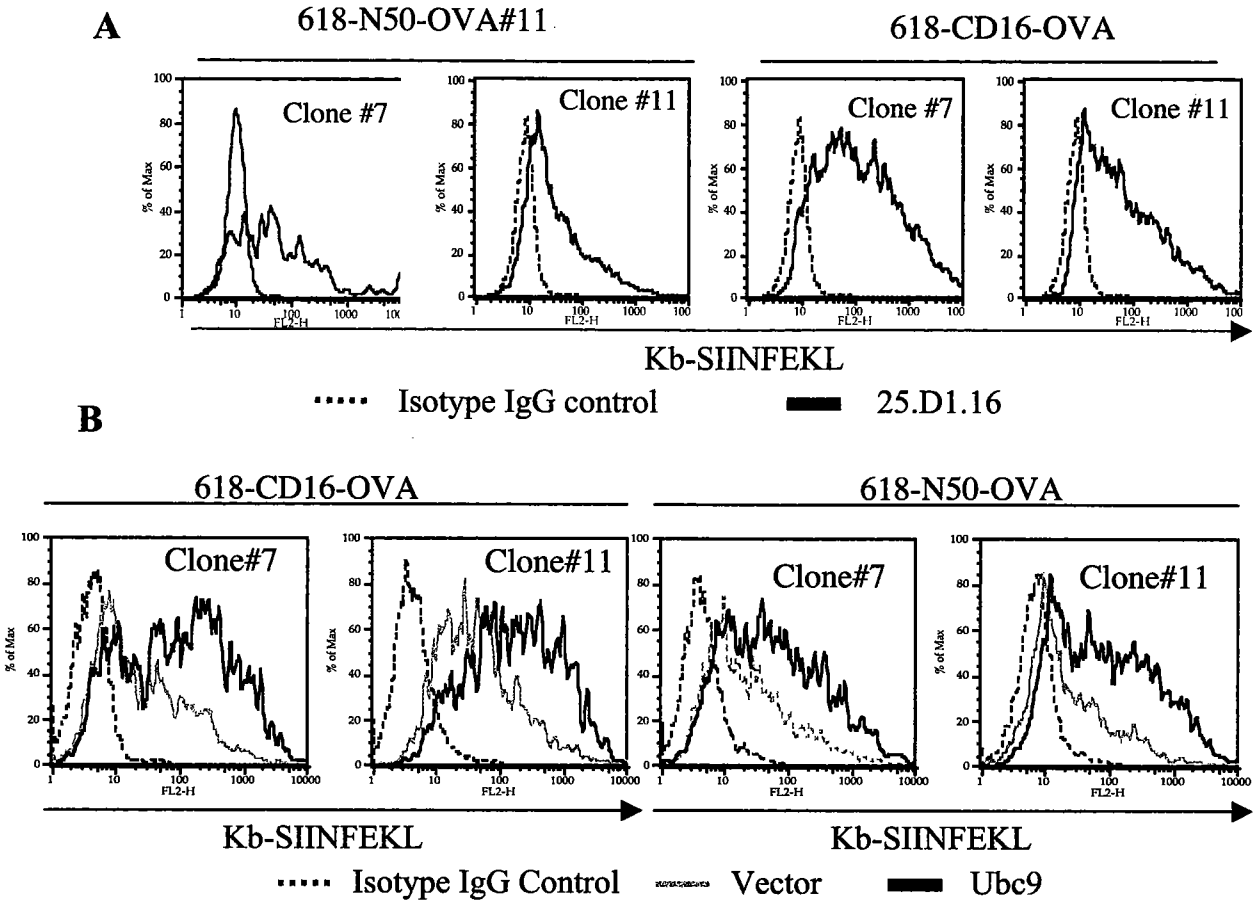


Fig 5. Ubc9 overexpression increases H-2Kb-SIINFEKL levels on COS-Kb cells stably transfected with ovalbumin. A. Two clones of COS-Kb stably expressing CD16-OVA (ER-targeted), and two clones expressing N50-OVA (cytosolic) ovalbumin were stained with anti-H-2Kb-SIINFEKL antibody 25.D1.16 and analyzed by flow cytometry. B. Stable ovalbumin transfectants were transfected with pTracer-CMV-Vector, or pTracer-CMV-Ubc9. Thirty-six hours after transfection, the cells were stained with 25.D1.16 and GFP+ populations were analyzed by flow cytometry.

The first steps in the MHC class I pathway are the transcription and translation of cellular and viral proteins. Because UBC9 has been found to regulate some transcription factors (148, 164, 169, 197-199), it seemed possible that UBC9 could be increasing H-2K^b-SIINFEKL levels by increasing the synthesis of antigen from the transfected constructs, even though the stable protein levels were similar (Figure 1D). I therefore performed protein loading experiments to bypass antigen synthesis. In these experiments, purified antigen is directly loaded into the cytosol of live cells, where it is processed by proteasomes, and the peptides generated are presented by MHC class I molecules. Cells transfected with pTracer-CMV-UBC9 or control plasmid were loaded with ovalbumin by hypertonic lysis of pinosomes (which delivers that antigen into the cytosol) and then grown in normal medium at 37°C. Surface H-2K^b-SIINFEKL levels were analyzed by flow cytometry at different time points (Fig 6). About 30 minutes after protein loading, H-2K^b-SIINFEKL complexes appeared on the cell surface, and reached their maximum level about 2 hours after protein loading. UBC9-transfected cells always expressed higher levels of H-2K^b-SIINFEKL than control vector-transfected cells. Therefore either UBC9 enhanced presentation from hypertonically-loaded protein, or UBC9 enhanced the uptake of proteins from extracellular medium. To know whether UBC9 increases protein uptake in protein loading experiments, I quantified the amount of protein loaded into cells by using a fluorescent-tagged protein (Cy5-conjugated goat anti-mouse IgG). FACS results showed that UBC9 did not increase protein uptake (Fig 7). Therefore UBC9 overexpression affects MHC class I antigen presentation step(s) after protein synthesis, and UBC9 does not increase H-2K^b-SIINFEKL levels by enhancing the transcription and synthesis of antigen.

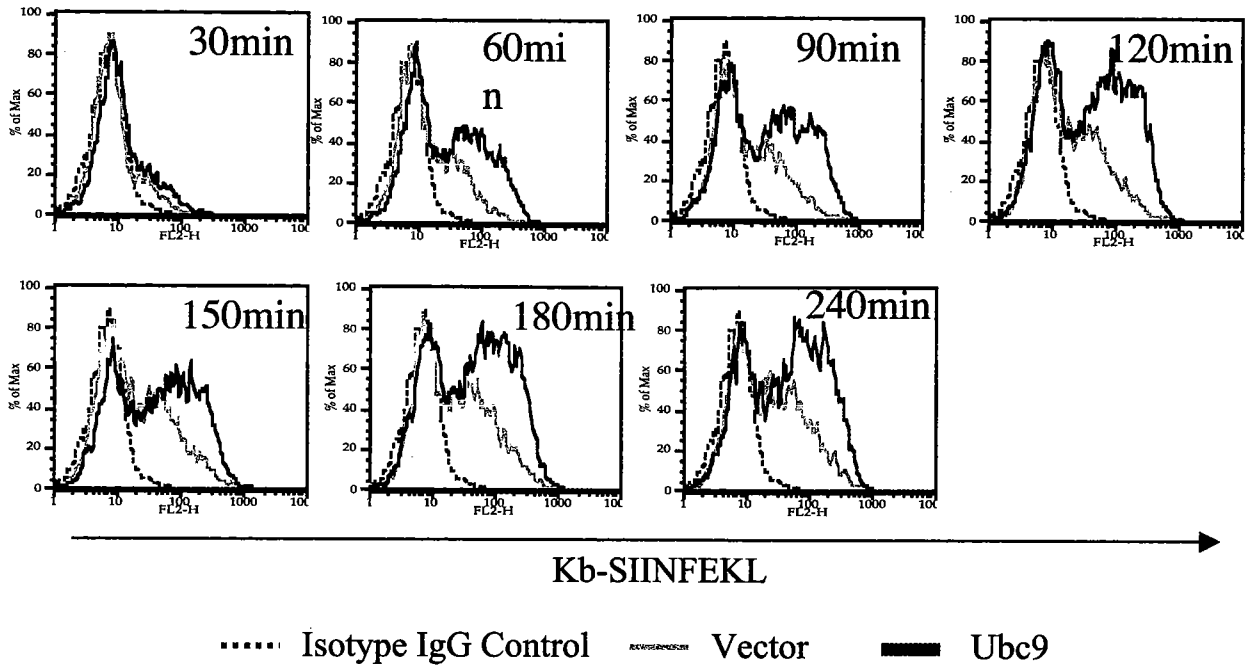


Fig 6. UBC9 overexpression increases antigen presentation in cell loaded with ovalbumin protein. COS-Kb cells were transfected with pTracer-CMV vector or pTracer-CMV-Ubc9, Thirty-six hours after transfection, the cells were loaded with ovalbumin (80mg/ml) by hypertonic lysis of pinosomes, incubated at 37°C, and stained with 25.D1.16 antibody at different time points. GFP+ cell populations were analyzed by flow cytometry.

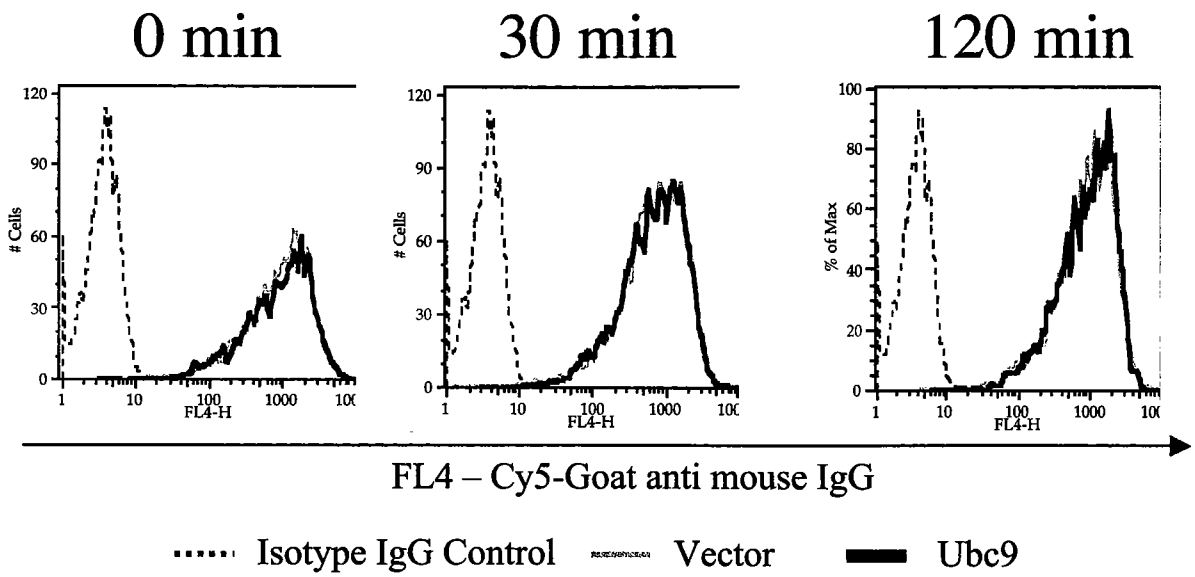


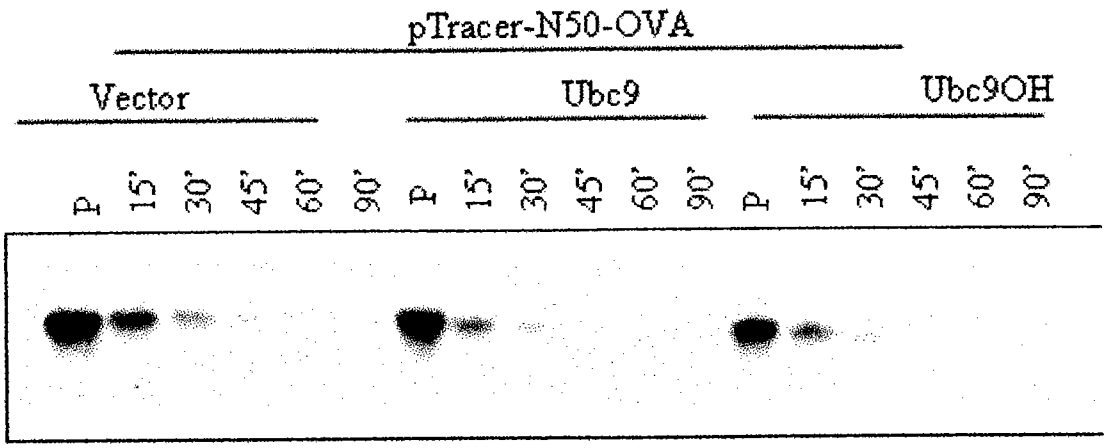
Fig 7. Ubc9 over-expression does not increase protein uptake. COS-Kb cells were transfected with pTracer-CMV-Vector or pTracer-CMV-Ubc9. Thirty-six hours after transfection, the cells were loaded with Cy5-conjugated goat anti-mouse IgG antibody by hypertonic lysis of pinosomes and incubated at 37°C. The cells were analyzed by flow cytometry at 0 min, 30 mins and 120 mins.

3.1.5. UBC9 does not increase MHC class I antigen presentation by increasing degradation of antigenic proteins

After antigens are synthesized, the next step in the MHC class I pathway is the degradation of the antigen into oligopeptides. Although most studies have shown that UBC9 sumoylates target proteins and modifies the function of the targeted proteins, there are some examples where UBC9 enhances the degradation of certain proteins (127, 128). To determine whether UBC9 up-regulates MHC class I antigen presentation by accelerating antigenic protein degradation, I performed pulse-chase experiments. Cos-K^b cells were co-transfected with plasmids expressing cytosolic ovabumin (N50-Ova) and either UBC9 or vector alone. Twenty eight hours after transfection, the cells were pulse-labeled with ^{35}S for 5 minutes and chased for 15 to 90 minutes. Lysates were immunoprecipitated with rabbit anti-ovalbumin antibody (Fig 8). UBC9 did not change the rate of degradation of ovalbumin. Therefore UBC9 does not increase MHC class I antigen presentation by regulating the overall rate of protein degradation.

3.1.6. Ovalbumin protein degradation is proteasome dependent

Most MHC class I presented peptides (including SIINFEKL from ovalbumin) are generated by proteasomes (8). It was possible that UBC9 was stimulating some other proteolytic pathway that was generating SIINFEKL. Therefore, I sought to verify that the increase in antigen presentation in UBC9-transfected cells was still proteasome-dependent. Cos-K^b cells were transfected with either control vector or UBC9, and hypotonically loaded with ovalbumin. After protein loading, the cells were grown in normal medium or in the presence of proteasome inhibitor MG132 for 8 hours (Fig 9). The proteasome inhibitor



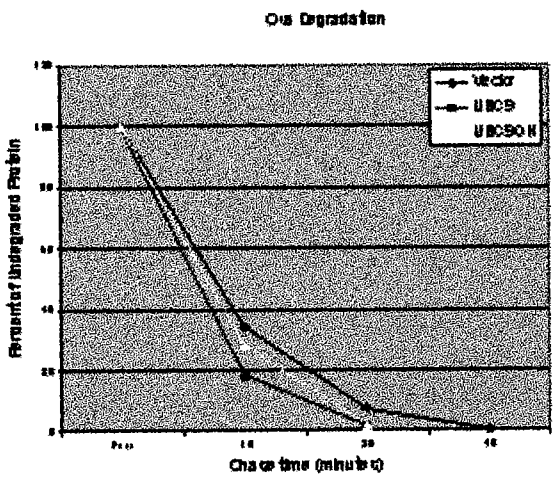


Fig 8. UBC9 and UBC9 OH do not strongly increase protein degradation. COS-Kb cells were co-transfected with pTracer-CMV-N50-OVA and pTracer-CMV vector, pTraver-CMV-UBC9 or pTracer-CMV-UBC9OH. Twenty-four hours after transfection, the cells are labeled with 35S-Met for 5 minutes and then chased for up to 90 minutes. Immunoprecipitation were done with rabbit anti-OVA antibody.

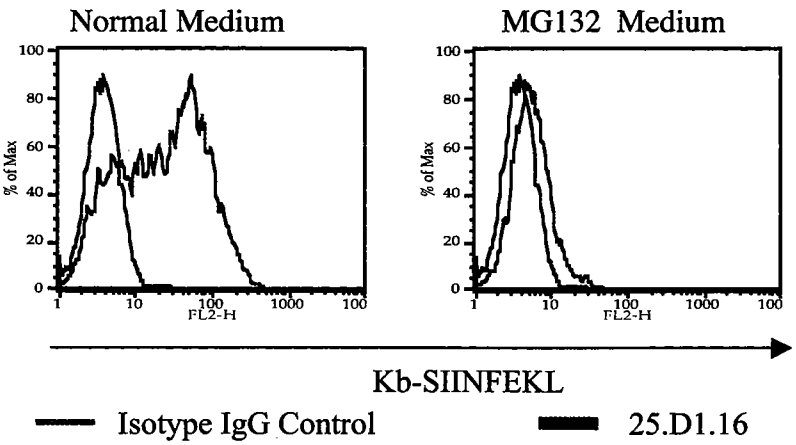


Fig 9. MHC class I antigen presentation is proteasome-dependant in the presence of UBC9. Cos-Kb cells were transfected with pTracer-Ubc9. Thirty-six hours after transfection, the cells were pre-incubated in MG13- containing medium (50μM) for 1 hour. The cells were loaded with ovalbumin (80mg/ml) by hypertonic lysis of pinosomes and incubated in the presence of MG132 for 3 hours. The cells were then stained with 25.D1.16 antibody and GFP+ cells were analyzed by flow cytometry.

almost completely inhibited MHC class I antigen presentation. This result demonstrates that even in the presence of UBC9, MHC class I antigen presentation is still proteasome dependent.

3.2. Analysis of UBC9 effects on oligopeptide constructs.

To further investigate whether UBC9 might enhance the generation of presented peptides by proteasomes, I used antigen constructs that did, or did not, require proteasomal cleavage for the generation of H-2K^b-SIINFEKL complexes. I made use of minigenes, consisting of the epitope SIINFEKL with short N- or C-terminal extensions, which our lab and others have shown to have specific requirements for antigen processing (47). For example, because carboxypeptidase activity is not present in the cytosol of most cells, any SIINFEKL precursors with even a single C terminal extension are dependent on proteasome activity for presentation of the mature epitope SIINFEKL. In contrast, SIINFEKL with short N-terminal extensions can bypass the proteasome, with cytosolic and ER aminopeptidases generating the mature SIINFEKL epitope. SIINFEKL can also be targeted directly to the ER with a signal sequence, so that cytosolic proteases and the TAP transporter are bypassed altogether. To rule out the possibility that UBC9 may affect MHC class I antigen presentation by affecting minigene transcription/translation, I also did experiments loading synthetic peptides directly into the cytosol, including N-terminal extended peptides (which require aminopeptidases for presentation), C-terminal extended peptides (which require proteasomes), and SIINFEKL peptide (which does not require any trimming).

3.2.1. UBC9 overexpression regulates proteasome-dependent presentation.

My previous results showed that UBC9 overexression increased H-2K^b-SIINFEKL levels transfected with all forms of protein. I asked whether UBC9 regulates proteasome-dependent presentation. When COS-K^b cells were transfected with C-terminal extended SIINFEKL minigene and UBC9 or control vector, UBC9 transfected cells expressed higher level of H-2K^b-SIINFEKL than control plasmid transfected cells (Fig 10A). Even when UBC9 transfected cells were loaded with SIINFEKLT (which has a single extra C-terminal flanking residue), the H-2K^b-SIINFEKL level was much higher than control plasmid transfected cells (Fig 10B). Considering the requirement for proteasomes for presentation of SIINFEKL from C-extended peptide, this suggested that proteasome-dependent presentation (or an event(s) downstream of the proteasome) is regulated by UBC9.

3.2.2. Ubc9 overexpression enhances N-terminal extended peptide presentation

Following the pathway of MHC class I antigen presentation, after longer oligopeptides are generated by proteasomes, the peptides are trimmed by aminopeptidases to generate the correct-sized peptides. Does UBC9 overexpression affect presentation of N-terminal-extended peptides? UBC9 only moderately increased levels of H-2K^b-SIINFEKL when UBC9 was transiently co-transfected with N-extended SIINFEKL minigene constructs (Fig 11). However, as with the cytosolic ovalbumin (N50-OVA) described above, it seemed possible that the high expression level of minigenes in transient transfections may saturate cell surface H-2K^b-SIINFEKL expression, disguising the effect of UBC9.

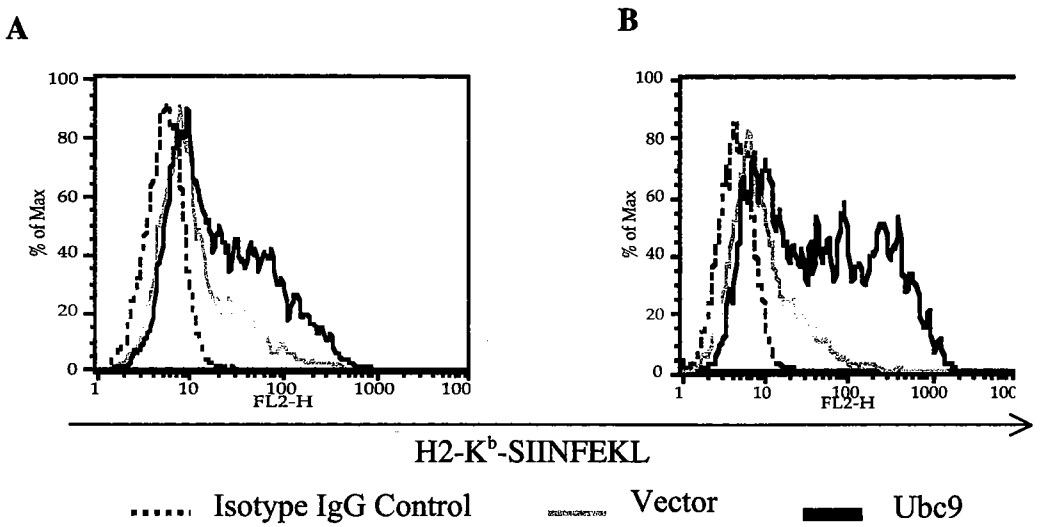


Fig 10. Ubc9 overexpression increases H-2K^b-SIINFEKL presentation from C-terminal-extended precursors. A) COS-Kb cells were co-transfected with C-terminal-extended SIINFEKL (C-15) and pTracer-CMV-Vector or pTracer-CMV-Ubc9. Twenty-eight hours after transfection, the cells were stained with 25.D1.16 antibody, and GFP +ve cells were analyzed by flow cytometry. (B) COS-K^b cells were transfected with pTracer-CMV-Vector or pTracer-CMV-Ubc9. Twenty-eight hours after transfection, the cells were loaded with SIINFEKLT (1mg/m)l, incubated for 2 hr, stained with 25.D1.16 antibody, and GFP +ve cells were analyzed by flow cytometry.

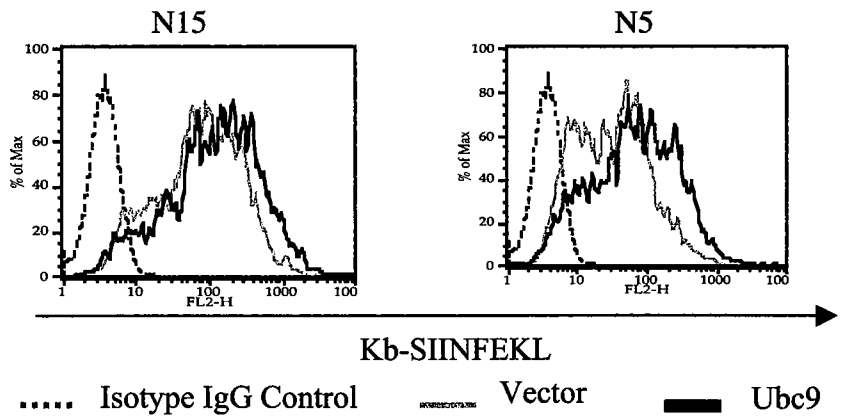


Fig 11. Ubc9 overexpression increases H-2Kb-SIINFEKL presentation from N-terminal-extended precursors. COS-Kb cells were co-transfected with pTracer-CMV-Ubc9 and SIINFEKL minigene with 5 (N5) or 15 (N15) extra amino acids at the N-terminus. Thirty-six hours after transfection, the cells were stained with 25.D1.16, and GFP +ve cells were analyzed by flow cytometry.

I therefore made tetracycline-inducible ovalbumin and SIINFEKL minigene expression plasmids, so that expression levels of the protein or peptides could be controlled. When Cos-Kb cells were co-transfected with pTet-ON-UBC9 and pBI-EGFP-CD16-OVA or pBI-EGFP-N50-OVAand induced with doxycyclin, the H-2K^b-SIINFEKL levels on UBC9-transfected cells were higher than on control vector transfected cells (Fig 12). This was consistent with our previous observations with UBC9 expression vectors, and confirmed that the inducible vectors could be used in these assays. I co-transfected pTet-ON-UBC9 with pBI-EGFP-N-terminal extended-minigene (N5-minigene, expressing LEQLESIINFEKL). Twenty-six hours after transfection, the cells were acid-washed to remove preexisting peptide-MHC complexes and grown in doxycyclin-containing medium for induction and stained for SIINFEKL-Kb complexes. Flow cytometry analysis showed that UBC9 overexpression slightly increased N-terminal extended peptide presentation (Fig 13a).

I also did similar experiments loading synthetic N-extended SIINFEKL peptides into the cytosol of cells. UBC9-transfected cells expressed higher level of H-2K^b-SIINFEKL than control plasmid transfected cells when loaded with N-extended SIINFEKL peptide (Fig 13b).

3.2.3. UBC9 overexpression increases SIINFEKL peptide presentation

UBC9 enhanced both N-extended and C-extended SIINFEKL presentation, so the next question is whether UBC9 had an effect on presentation of SIINFEKL itself. UBC9 did not increase H-2K^b-SIINFEKL levels in cells transfected with either constitutive or inducible expression plasmids (Fig 14A and 14B). Interestingly, however, when synthetic SIINFEKL

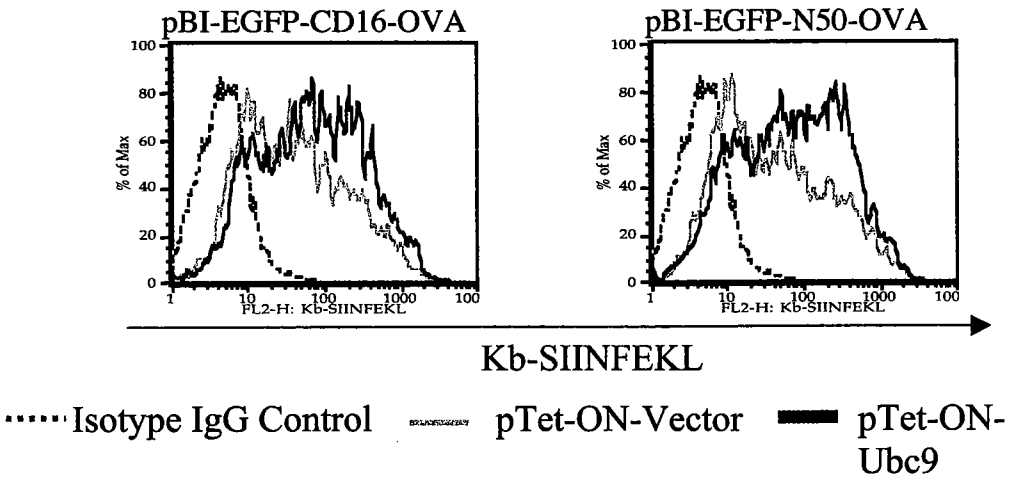


Fig 12. Ubc9 increases H-2Kb-SIINFEKL levels from cells inducible expressing ovalbumin. COS-Kb cells were co-transfected with pTet-ON-Ubc9 and pBI-EGFP-CD16-OVA, or pTet-ON-Ubc9 and pBI-EGFP-N50-OVA. Twenty hours after transfection, the cells were acid-washed and grown in DOX (20μg/ml) containing medium. After a further 4 hours incubation, the cells were stained with 25.D1.16 antibody, and GFP +ve cells were analyzed by flow cytometry.

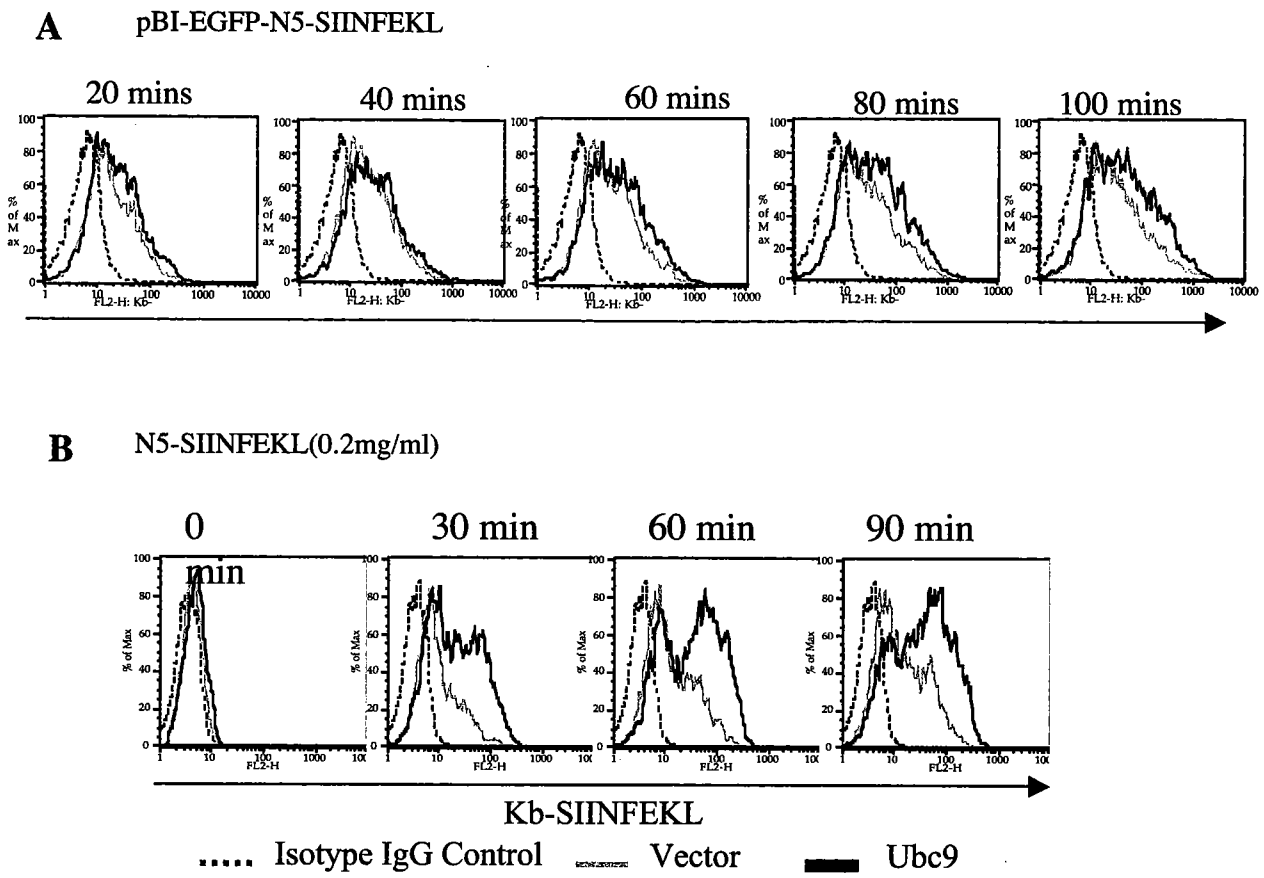


Fig 13. Ubc9 overexpression increases H-2Kb-SIINFEKL levels from N-terminal extended SIINFEKL precursors. A: COS-Kb cells were co-transfected with pTet-ON-Ubc9 and pBI-EGFP-N5-SIINFEKL. Twenty hours after transfection, the cells were acid-washed and grown in DOX (20μg/ml) containing medium. At various time points the cells were stained with 25.D1.16 antibody, and GFP +ve cells were analyzed by flow cytometry. B: COS-K^b cells were transfected with pTracer-CMV-Vector or pTracer-CMV-Ubc9. Twenty-eight hours after transfection, the cells were loaded with N-extended LEQLESIINFEKL (0.2mg/ml). At various time points the cells were stained with 25.D1.16 and GFP +ve cells were analyzed by flow cytometry.

peptide was hypertonically loaded directly into the cells (with subsequent acid washing to remove peptide bound directly to surface-resident H-2K^b), UBC9-transfected cells did express higher levels of H-2K^b-SIINFEKL than control plasmid-transfected cells (Fig 14C). The inconsistency may reflect the difference between the minigene-encoded sequence and the synthetic SIINFEKL peptide, in which the minigene expression product has an extra non-native residue, Met, at the N-terminus as the initiation codon, so that some aminopeptidase trimming is required. To know whether this additional N-terminal amino acid has any effect on peptide processing, we made a MIINFEKL minigene expression plasmid; the translation product of this cDNA will be an eight residue peptide that will require no further trimming. The affinity of MIINFEKL to H-2K^b molecules is similar to SIINFEKL, and recognition of MIINFEKL-H-2K^b by 25.D1.16 is smilar to that of SIINFEKL-H-2K^b (1). When the MIINFEKL minigene was co-transfected with UBC9, more H-2K^b-MIINFEKL complexes on cell surface were observed than in control vector-transfected cells (Fig 14D), which was consistent with the SIINFEKL peptide-loading experiments results. Although we do not know why the initiating methionine on MSIINFEKL has such an effect on peptide processing and presentation, UBC9 clearly can increase antigen presentation of SIINFEKL from all forms of ovalbumin antigens.

3.2.4. UBC9 affects ER processes

Peptides generated in the cytosol are transported into ER via an ER-membrane complex, TAP1/TAP2. In the ER, peptides are bound to empty MHC class I molecules. Many ER proteins are involved in this process, including ER chaperones such as calnexin, calreticulin, and ERp57, and tapasin (98) (99), which tethers empty MHC class I molecules

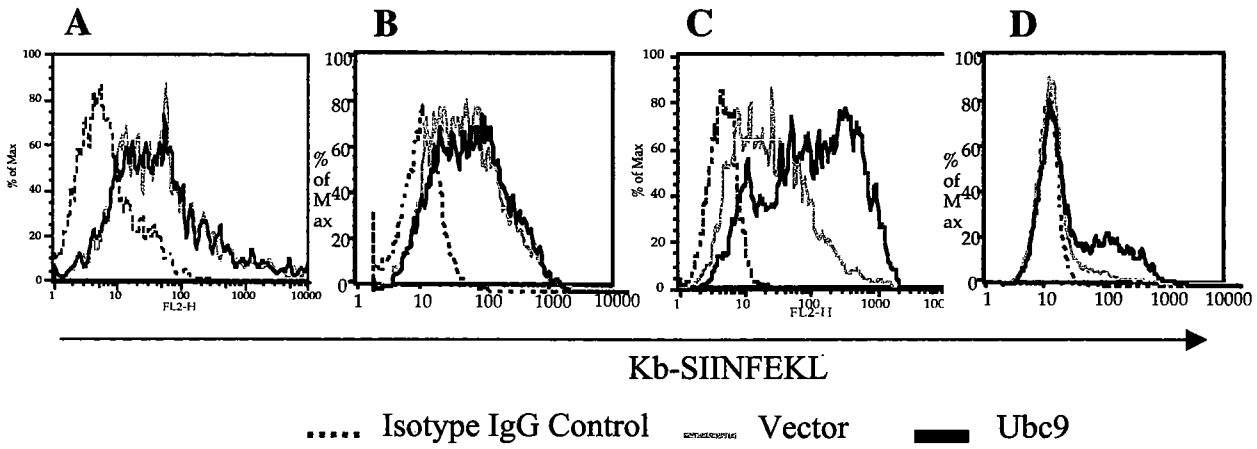


Fig 14. Ubc9 over-expression increases H-2K^b-SIINFEKL levels on cells loaded with SIINFEKL peptide or transfected with MIIFEKL minigene. A. COS-Kb cells stably expressing MSIINFEKL were transfected with control vector or UBC9. Twenty-four hours after transfection the cells were stained with 25.D1.16 antibody and GFP +ve cells were analyzed by flow cytometry. B: COS-K^b cells were co-transfected with pTet-ON-Ubc9 and pBI-EGFP-SIINFEKL minigene. Twenty-four hours after transfection, the cells were acid-washed and grown in DOX (20μg/ml) containing medium. C: Empty vector or UBC9-transfected cells were loaded with SIINFEKL peptide (0.1mg/ml), **acid washed and incubated for 2 hours, and then stained with 25.D1.16 antibody.** D: COS-K^b cells were co-transfected with MIINFEKL minigene and pTracer-CMV-Vector or pTracer-CMV-Ubc9. Twenty-four hours after transfection the cells were stained with 25.D1.16 antibody and GFP +ve cells were analyzed by flow cytometry.

and TAP1/TAP2 complex together (35, 200). To know whether UBC9 affects MHC class I antigen presentation by regulating components in ER lumen, I made an inducible ER-targeted SIINFEKL minigene expression plasmid, in which the SIINFEKL epitope is translocated into the ER lumen by a signal sequence. When COS-K^b cells were co-transfected with UBC9 and the ER-targeted minigene and induced with tetracyclin, UBC9 increased H-2K^b-SIINFEKL levels on the surface (Fig 15), suggesting that UBC9 may affect MHC class I antigen presentation by regulating ER components.

3.2.5. ICP47 does not affect UBC9 function in regulating ER-targeted minigene presentation

Presentation of SIINFEKL from the ER-targeted minigene should be TAP-independent. However, it was also possible that a significant amount of the presentation from this construct was from peptide that was mistranslated or mistargeted and that never reached the ER (e.g., defective ribosomal products [DRiPs] or mistargeted molecules). This form of presentation would not require ER components to be altered, and would be TAP-dependent. It is also possible that peptides are retrotranslocated from the ER into the cytosol and processed there. To rule out the possibility that UBC9 affects ER protein presentation by affecting cytosolic factors, I used the herpes simplex virus protein ICP47 to study the function of UBC9 on ES-minigene presentation. ICP47 inhibits MHC class I antigen presentation by blocking the TAP1/2 complex (110, 111). Twenty-four hours before transfection, COS-K^b cells were infected with a replication-defective recombinant adenovirus that expresses ICP47 (110, 111). As expected, ICP47 efficiently inhibited MHC class I presentation from full-length ovalbumin (Figure 16A), and markedly decreased

pBI-EGFP-ES-SIINFEKL

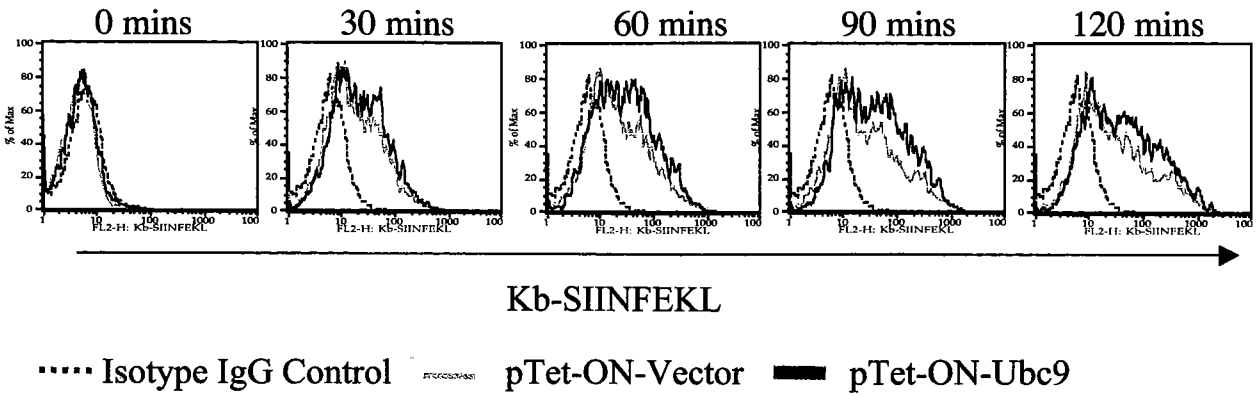


Fig 15. Ubc9 increases H-2Kb-SIINFEKL levels on cells transfected with ER-targeted SIINFEKL minigenes. COS-Kb cells were co-transfected with pBI-EGFP-ES-SIINFEKL (SIINFEKL targeted to the ER by a signal sequence) and pTracer-CMV vector or pTracer-CMV-Ubc9. Twenty hours after transfection, the cells were acid-washed and grown in DOX (20μg/ml) containing medium. At various times the cells were stained with 25.D1.16 antibody and GFP +ve cells were analyzed by flow cytometry

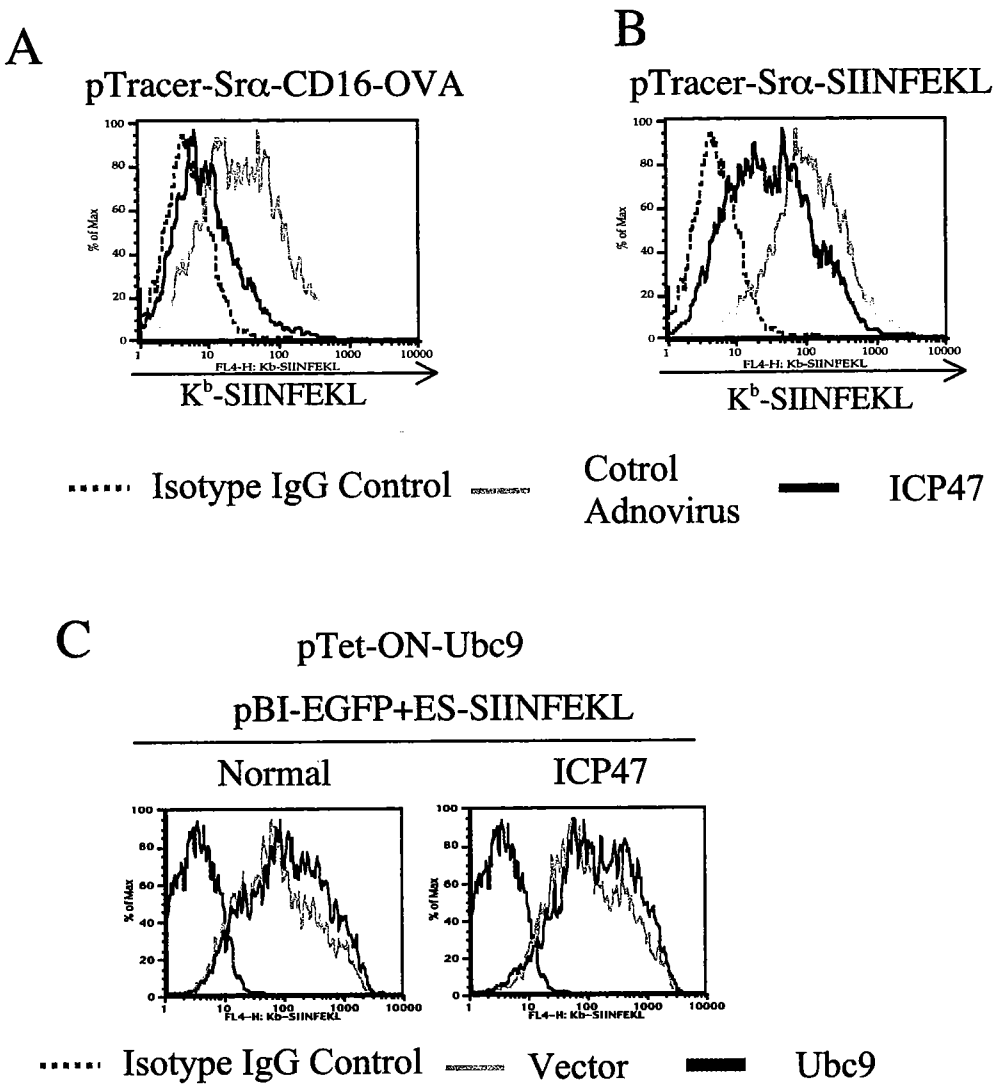


Fig 16. ICP47 does not inhibit Ubc9's up-regulation of H-2Kb-SIINFEKL on cells transfected with ES-minigene. Control adenovirus, or Ad-ICP47, -infected COS-Kb cells were transfected with different pTracer-SRα-CD16-OVA (A), pTracer-SRα-minigene (B), or pBI-EGFP-ES-minigene with pTet-ON-Vector/pTet-ON-Ubc9 (C). Twenty-six hours after transfection, the cells were stained with 25.D1.16 antibody (A and B), or the cells were acid-washed and grown in DOX (20μg/ml) containing medium for 4 hours, and then stained with 25.D1.16 antibody (C). GFP+ cell populations were analyzed by flow cytometry.

SIINFEKL minigene presentation (Fig 16B), but did not affect presentation of SIINFEKL from the ER-targeted minigene (Figure 16C), which bypasses the TAP blockade. Even in the presence of ICP47, UBC9 increased H-2K^b-SIINFEKL level when COS-K^b cells were transfected with ER-targeted minigene (Fig 16C). These results suggested that UBC9 affects MHC class I antigen presentation by regulating ER components.

3.2.6. UBC9 overexpression increases H-2K^b-SIINFEKL level on E36-K^b cells transfected with all forms of SIINFEKL minigenes

In addition to the experiments using Cos-K^b cells described above, I also used H-2K^b-transfected hamster cells, E36-K^b, to study the function of UBC9 in MHC class I antigen presentation. In these experiments, E36-K^b cells were transfected with UBC9 and several SIINFEKL minigenes, including N15-minigene (encoding LVLLPDEVSGLEQLESIINFEKL), N5-minigene (encoding LEQLESIINFEKL), C5-minigene (encoding SIINFEKLTEWTS), C15-minigene (encoding SIINFEKLTEWTSSNVMEERKIK), ER-targeted SIINFEKL minigene, and MSIINFEKL minigene. Forty-eight hours after transfection, the cells were analyzed by flow cytometry (Fig 17). UBC9 overexpression increased cell surface H-2K^b-SIINFEKL levels on E36-K^b cells transfected with all forms of minigenes, suggesting a role of UBC9 in regulating MHC class I antigen presentation. Notably, in E36-K^b cells, MSIINFEKL minigene presentation was augmented by UBC9, which is in contrast to the COS-K^b transient tranfection experiments described above. Not as shown in figure 14; in which SIINFEKL minigene was unaffected by UBC9 whether inducible or constitutive. It was consistent with the peptide and the MIINFEKL results.

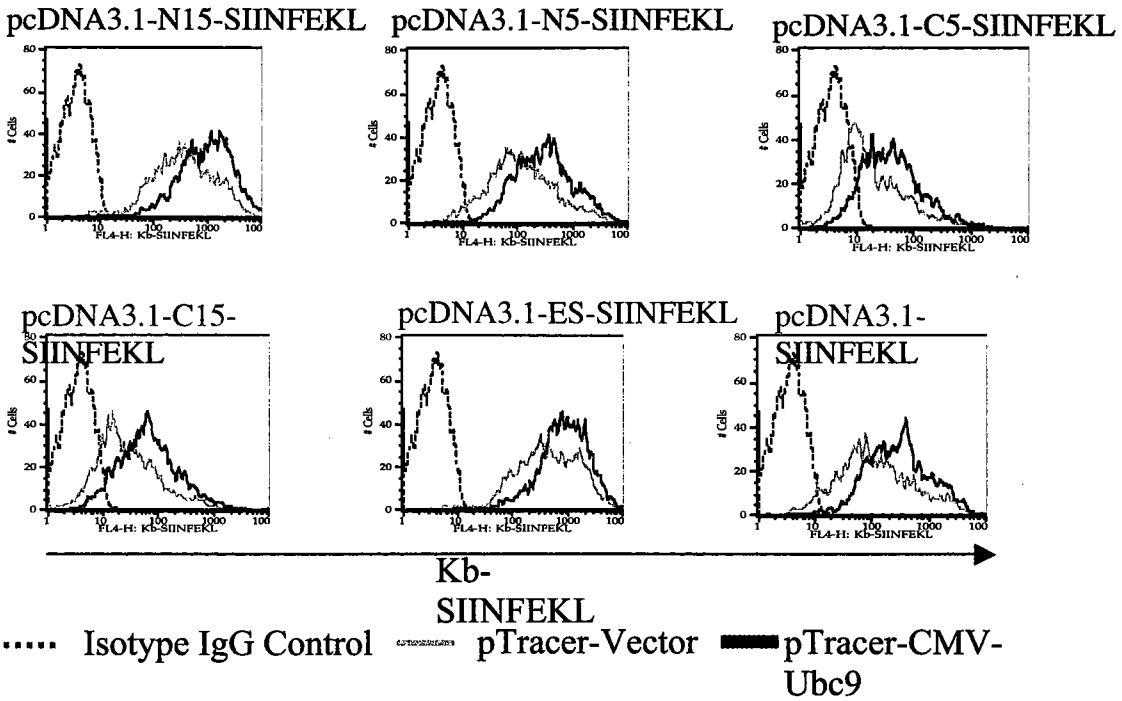


Fig 17. Ubc9 overexpression increases H-2Kb-SIINFEKL level on E36.Kb cells transfected with several SIINFEKL precursors. E36-Kb cells were co-transfected with combinations of plasmids as noted. Forty-eight hours after transfection, the cells were stained with 25.D1.16 antibody, and GFP+ cell populations were analyzed by flow cytometry. N15-SIINFEKL and N5-SIINFEKL: N-extended SIINFEKL. C5-SIINFEKL and C15-SIINFEKL: C-extended SIINFEKL. ES-SIINFEKL: SIINFEKL targeted to the ER by a signal sequence. SIINFEKL: MSIINFEKL minigene.

3.3. UBC9 does not regulate MHC class I antigen presentation by regulating aminopeptidases or protecting peptide from degradation.

3.3.1. UBC9 does not regulate aminopeptidases

Proteasomes generate peptides whose length is 4-24 amino acid residues (43). The C-terminus of peptides presented by MHC class I molecules must be generated by proteasomes, but N-terminal amino acids can be trimmed from longer peptides to generate the correct N-terminus (29, 46, 47). These longer peptides are trimmed by aminopeptidases, which exist both in the cytosol and the ER lumen. So far, at least four aminopeptidases have been found to play a role in trimming precursors of antigenic peptides: leucine aminopeptidase (48), bleomycin hydrolase (BH), puromycin sensitive aminopeptidase (PSA) (49, 201), and ER aminopeptidase 1 (ERAP1) (1, 91), also termed ER aminopeptidase associated with antigen processing (ERAAP) (90).

My peptide-loading experiments showed that UBC9 increased H-2K^b-SIINFEKL levels on cells loaded with all forms of SIINFEKL and its precursors. While the most dramatic effect was seen on C-terminal extended peptides, which require the proteasome for SIINFEKL generation, significant increases were also seen on N-extended peptides, which do not depend on the proteasome for SIINFEKL generation. The results suggested that even if UBC9 regulates MHC class I antigen presentation by regulating the activities of aminopeptidases, aminopeptidases are not the only affected step.

It has been reported that one aminopeptidase, BH, binds to Ubc9 (202), suggesting that Ubc9 may regulate H-2K^b-SIINFEKL by regulating aminopeptidase activity. Consistent with this possibility, I found that UBC9 increased levels of BH protein when the genes were co-transfected (Fig 18). To more specifically examine the effect of UBC9 on specific aminopeptidases, COS-K^b cells were co-transfected with ovalbumin and either BH or PSA. Both BH and PSA increased H-2K^b-SIINFEKL levels when co-transfected with full length ova (Fig 19) , though PSA's effect was very slight. We co-transfected COS-K^b cells with BH, an inactive mutant of BH, UBC9, or a control gene, ORP150. Twenty-eight hours after transfection, the cells were loaded with ovalbumin protein, and analyzed by flow cytometry. Co-transfections with UBC9 and wild type or mutant BH had no more effect on H-2K^b-SIINFEKL levels than did transfection with UBC9 alone (Fig 20), presumably because BH levels were now not limiting and/or peptide amounts generated are saturating. Therefore, part of the effect of UBC9 on antigen presentation of N-extended precursors may be through its increasing BH. However, this cannot account for the effect of UBC9 on the C-terminal-extended SIINFEKL precursors, or (since BH is a cytosolic enzyme) on the ER-targeted SIINFEKL.

3.3.2. UBC9 does not regulate MHC class I antigen presentation by protecting peptide from degradation

The finding that UBC9 increased H-2K^b-SIINFEKL level on cells loaded with all forms of SIINFEKL peptides suggest another mechanism by which UBC9 could increase antigen presentation: by decreasing the destruction of peptides. For example, an endopeptidase,

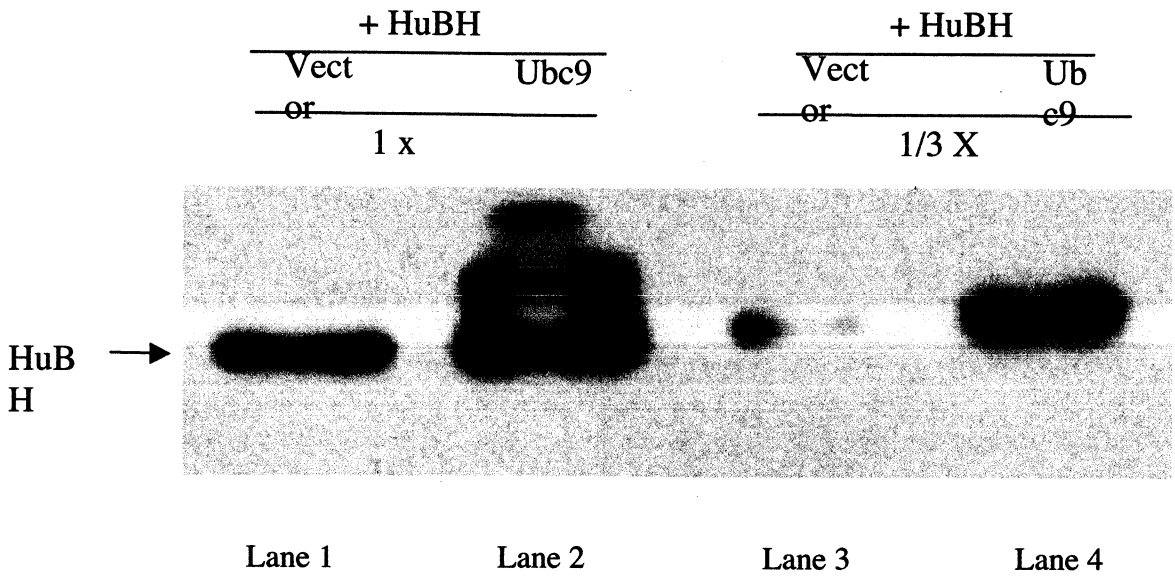


Fig 18. Ubc9 increases the levels of co-transfected bleomycin hydrolase. Cos-K^b cells were transfected with pcDNA3.1/HisB-Xpress-HuBH and Ubc9 or Vector. Twenty-four hours after transfection, the cells are lysed and whole cell lysates are subjected to western blot with anti-X-press antibody. Lane 1: HuBH+pTracer vector; lane 2: HuBH+pTracer-UBC9. Lane 3 and lane 4 are load with 1/3 of the amount of lysates of lane1 and lane 2 respectively.

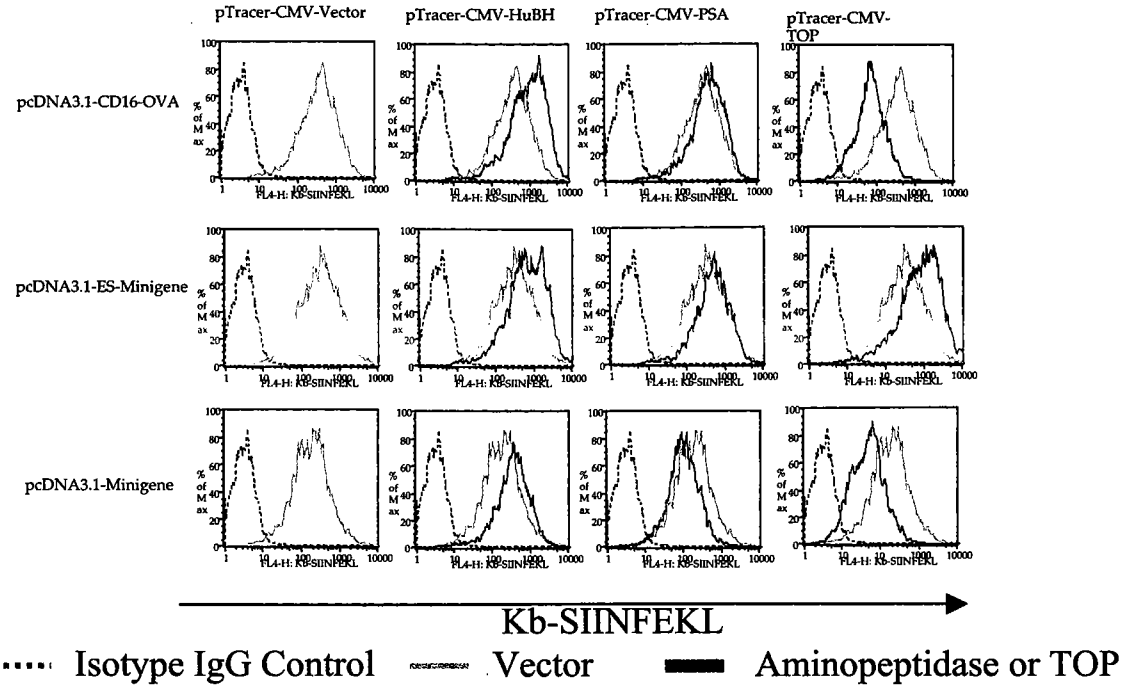


Fig 19. Overexpression of peptidases affects MHC class I antigen presentation. COS-Kb cells were co-transfected with plasmids expressing no insert (left column), bleomycin hydrolase (second column), puromycin-sensitive aminopeptidase (third column), or thimet oligopeptidase (rightmost column) and the indicated ovalbumin plasmids. Forty-four hours after transfection, the cells were stained with 25.D1.16 and GFP +ve cells were analyzed by flow cytometry.

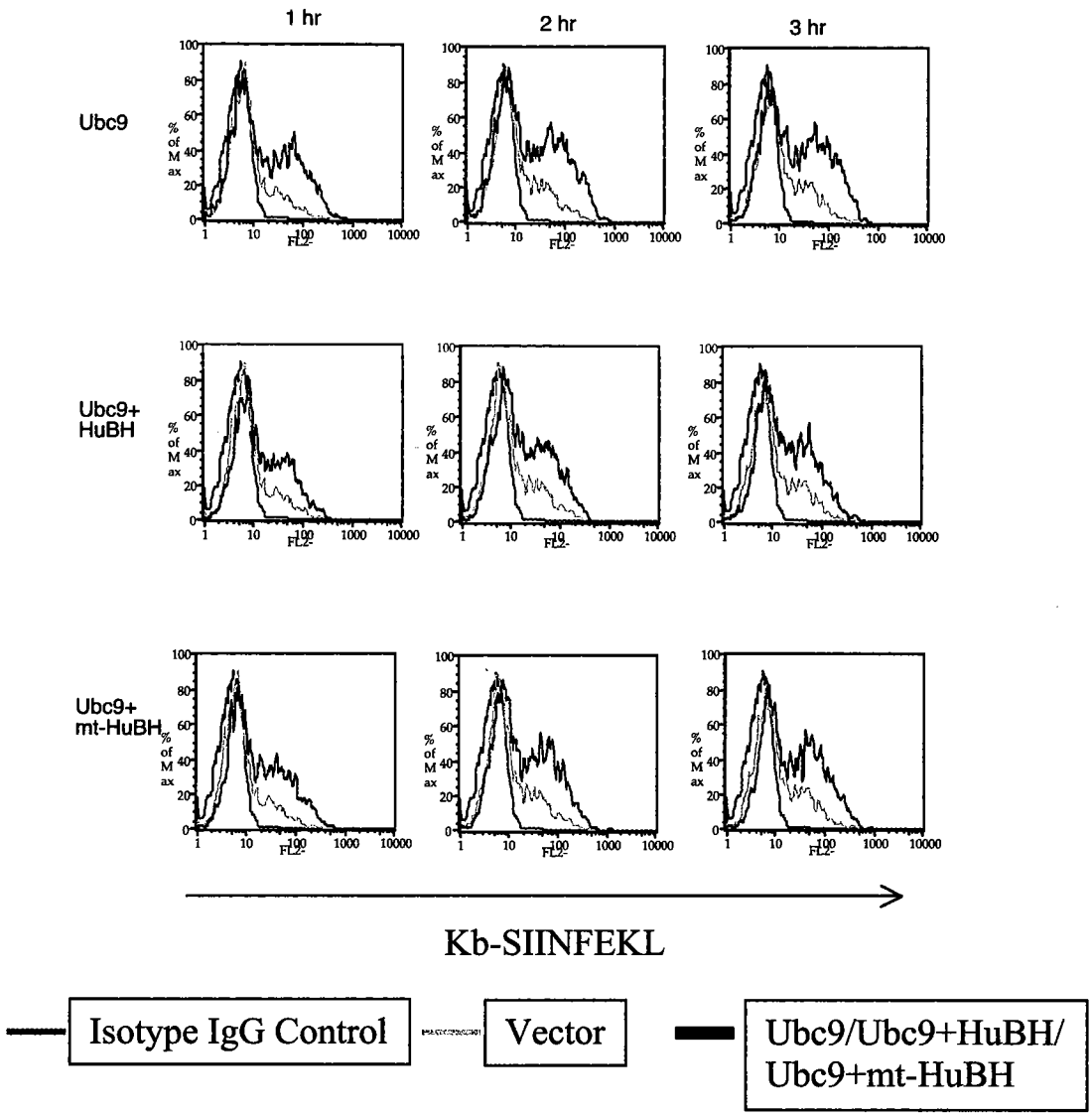


Fig 20. Ubc9 does not further increase H-2Kb-SIINFEKL levels in cells transfected with bleomycin hydrolase. COS-Kb cells were transfected with lasmids expressing Ubc9, Ubc9 and BH, or Ubc9 and an inactive mutant of BH. Twenty-eight hours after transfection, the cells were loaded with ovalbumin, incubated at 37°C, and stained with 25.D1.16 antibody at different time points. GFP +ve cells were analyzed by flow cytometry.

thimet oligopeptidase (TOP), has been found to destroy peptides and thereby reduce MHC class I antigen presentation (Fig 19) (195). Inhibition of TOP (69), or other destructive peptidases (1) increases MHC class I presentation by reducing peptide destruction. Therefore, we asked if UBC9 might protect peptides from degradation. In this experiment, I used COS7 cells, rather than COS-Kb, in order to avoid the complication of SIINFEKL binding to H-2K^b and being protected from peptidases. COS7 cells were transfected with control vector or UBC9 plasmid. Thirty-six hours after transfection, cytoplasmic extracts were prepared and ESIINFEKL peptide was incubated with the extracts and analyzed by RP-HPLC at intervals. Peptide was degraded at very similar rates from extracts of control and UBC9-transfected extracts, showing that UBC9 did not protect peptides from degradation (Fig 21).

We also examined peptide stability in intact cells. pTracer-CMV-vector or pTracer-CMV-UBC9 plasmid-transfected COS7 cells were hypertonically loaded with SIINFEKL peptide and grown at 37 °C. At different time points peptides were extracted with 10% TCA or TFA, and used for antigen presentation assays (Fig 22). Again, UBC9 did not protect peptides from degradation.

3.4. UBC9 overexpression increases the levels of several proteins in the antigen presentation pathway

3.4.1. UBC9 upregulates immunoproteasome components

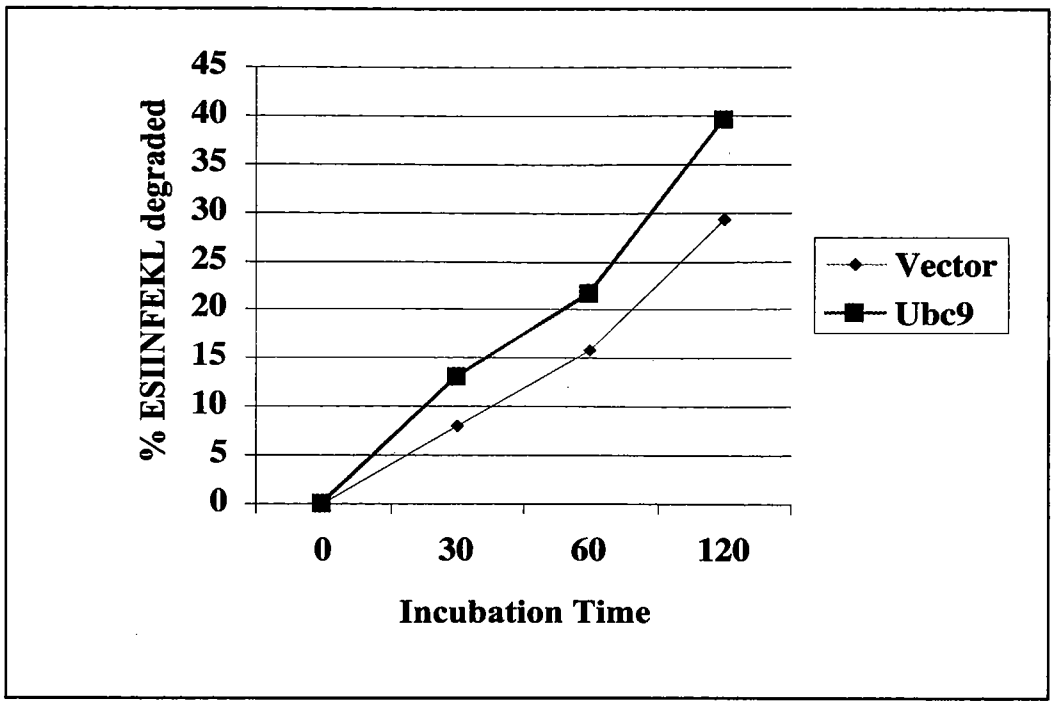


Fig 21. Ubc9 overexpression does not protect peptides from degradation. COS-7 cells were transfected with pTracer-CMV-Vector or pTracer-CMV-Ubc9. Thirty-six hours after transfection, the cytosol was extracted as described in the Materials and Methods and used for ESIINFEKL peptide digestion. Peptide degradation rates were analyzed by HPLC on a C18 column.

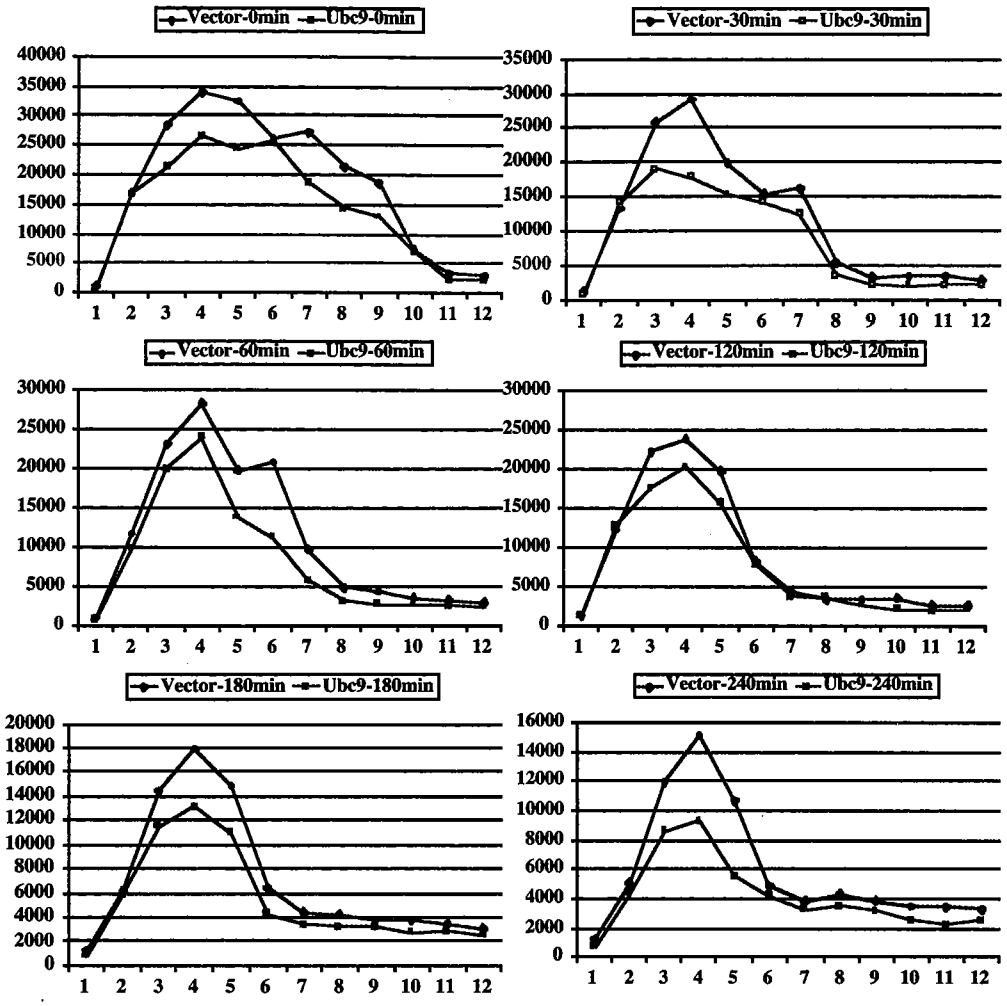


Fig 22. Ubc9 overexpression does not protect peptides from degradation. COS-7 cells were transfected with pTracer-CMV-Vector or pTracer-CMV-Ubc9. Twenty-eight hours after transfection, the cells were loaded with SIINFEKL peptide, acid-washed, and incubated at 37°C. At various times the cells were lysed with 5% TFA, and peptides were extracted and used to stimulate the H-2Kb-SIINFEKL-specific hybridoma RF33.70 as described in the Methods and Materials. Shown is the IL-2 release from RF33.70 as measured by incorporation of ^{3}H-thymidine by the IL2-dependent cell line CTLL.

UBC9 increased H-2K^b-SIINFEKL levels more from C-terminal-extended minigenes than from N-terminal extended constructs or from SIINFEKL itself. Since C-terminally-extended peptides require proteasomes for processing and presentation on MHC class I molecules (46, 47), this finding suggested that UBC9 might be affecting proteasomes. Although we found that UBC9 did not change the protein degradation rate (Figure 8), it was possible that UBC9 may modify the components of the proteasome, or regulate the activity of proteasome components in ways that alter the cleavage pattern of antigens, so that proteasomes more efficiently generate antigenic peptides from degraded proteins. It has been shown that immunoproteasomes, in which the catalytic β1, β2, and β5 subunits have been replaced by LMP2, LMP7, and MECL-1, generate different peptides than do constitutive proteasomes, releasing from ovalbumin 2-4 times more of certain N-extended versions of SIINFEKL (29) that can be processed to the mature epitope, so that more antigenic peptides are ultimately generated per molecule of protein degraded. To know whether UBC9 induced LMP2, LMP7 and MECL-1, COS-K^b cells overexpressing UBC9 were stained with mouse anti-UBC9 and with anti-LMP2, anti-LMP7 or anti-MECL-1 antibodies and examined by fluorescent microscopy (Fig 23A, 23B and 23C). The cells were transfected under conditions so that only a minority of cells expressed UBC9, so that neighboring untransfected cells (not staining with anti-UBC9) could be used as controls. In UBC9-transfected cells, the LMP2, LMP7 and MECL-1 staining was markedly stronger than in adjacent untransfected cells. This was not because of spectral overlap between FITC and rhodamine, because appropriate filters were used and several other proteins (e.g. calnexin and calreticulin), stained as controls, showed no change in intensity in UBC9

expressing cells (see below). These observations suggested that UBC9 may increase the amount of some immunoproteasome components.

3.4.2. UBC9 increases TAP1 and Tapasin protein levels

Our transfection and peptide loading experiments showed that UBC9 overexpression increased cell surface H-2K^b-SIINFEKL levels on COS-K^b cells loaded with SIINFEKL peptide (Figure 14C), suggest that it may also regulate factors downstream of proteolytic processing, such as TAP or other ER factors. Immunofluorescence experiments showed that UBC9 upregulated TAP1 and tapasin (Fig 23D and 23E), but had no effect on calnexin (Fig 23F), calreticulin (Fig 23G), ERp57 (Fig 23H), or PDI (Fig 23I), or on the cytosolic protein leucine aminopeptidase (LAP) (Fig 23J).

3.4.3. UBC9 inhibits degradation of some proteins in the MHC class I presentation pathway

The results above established that UBC9 increased the level of several proteins (LMP2, LMP7, MECL-1, TAP1 and tapasin) that are associated with antigen presentation. The increase in these proteins could be due to changes in their transcription or translation, or may be due to post-translational effects. To know whether UBC9 increased these components at the level of transcription, Real-Time PCR was used to analyze the levels of MECL1 mRNA. Although MECL-1 protein was markedly upregulated by UBC9 overexpression (Fig 23A), there was no difference in UBC9 mRNA levels between vector and UBC9-transfected cells (Fig 24), suggesting that UBC9 does not affect transcription of this gene, and by extension the other genes whose protein levels increased.

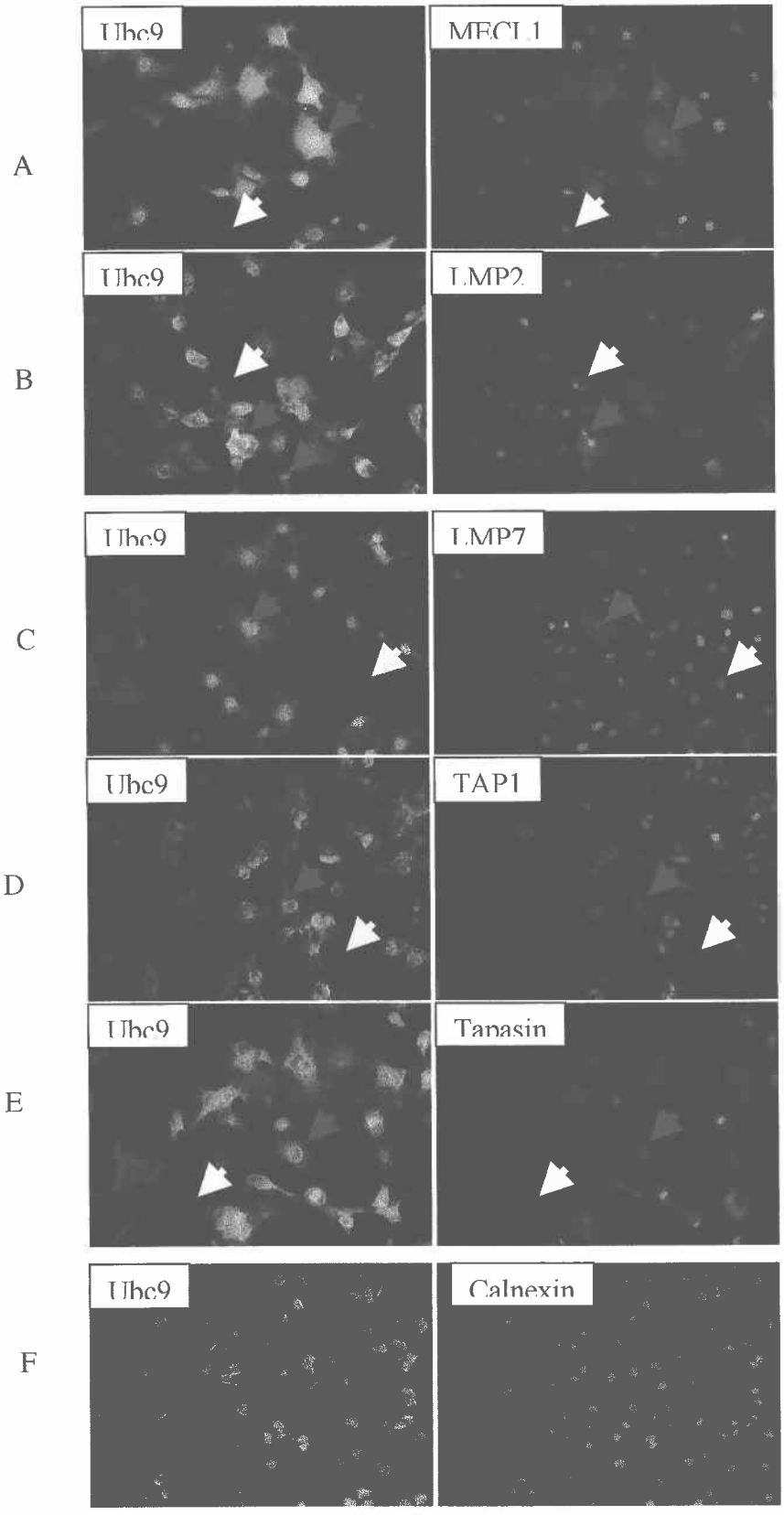


Fig 23 A-F. (Figures 23 G-J on following page)

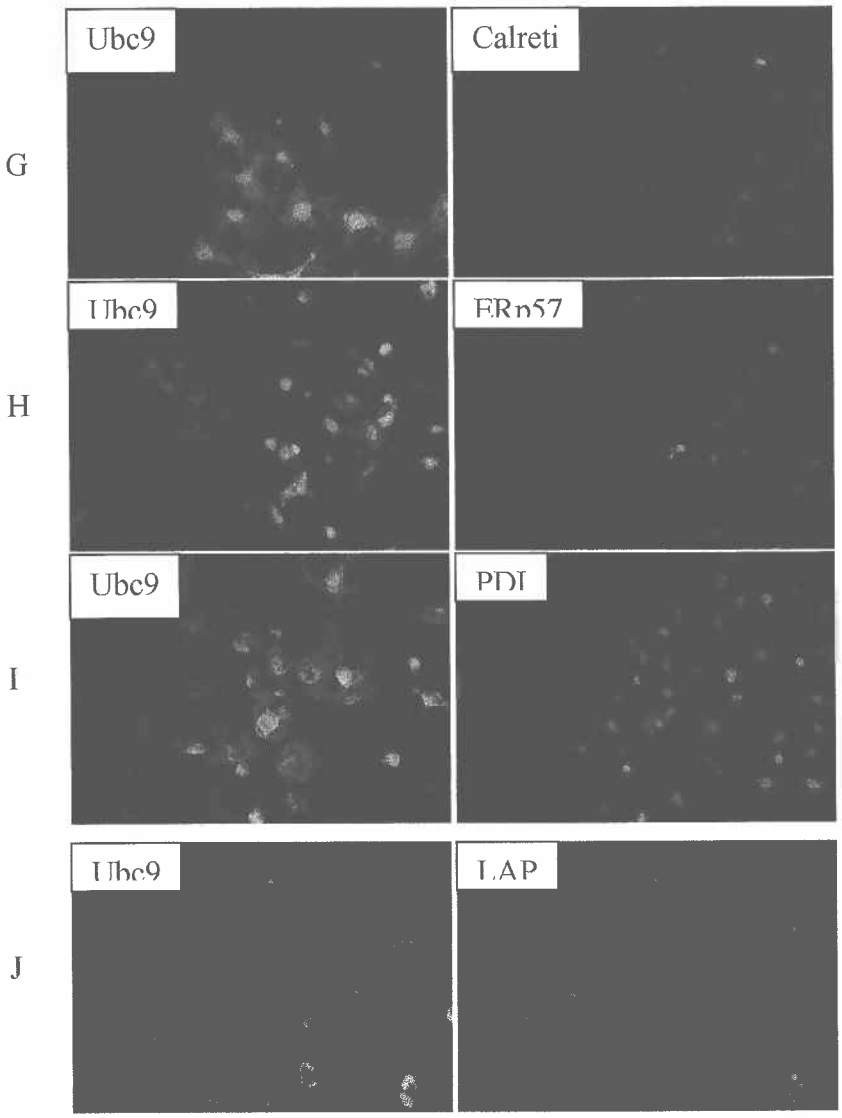


Fig 23. Ubc9 over-expression increases MECL-1, LMP2, LMP7, TAP1, and tapasin levels. Cos-Kb cells were transfected with a plasmid expressing Ubc9. Thirty hours after transfection, the cells were stained with anti-UBC9 conjugated to FITC, and various other antibodies as indicated conjugated to Texas Red. The cells were examined by confocal microscopy as described in the Materials and Methods. Compare UBC9 transfected cells (red arrow) and un-transfected cells (yellow arrow), UBC9 overexpression specifically increase MECL1, LMP2, LMP7, TAP1 and Tapasin

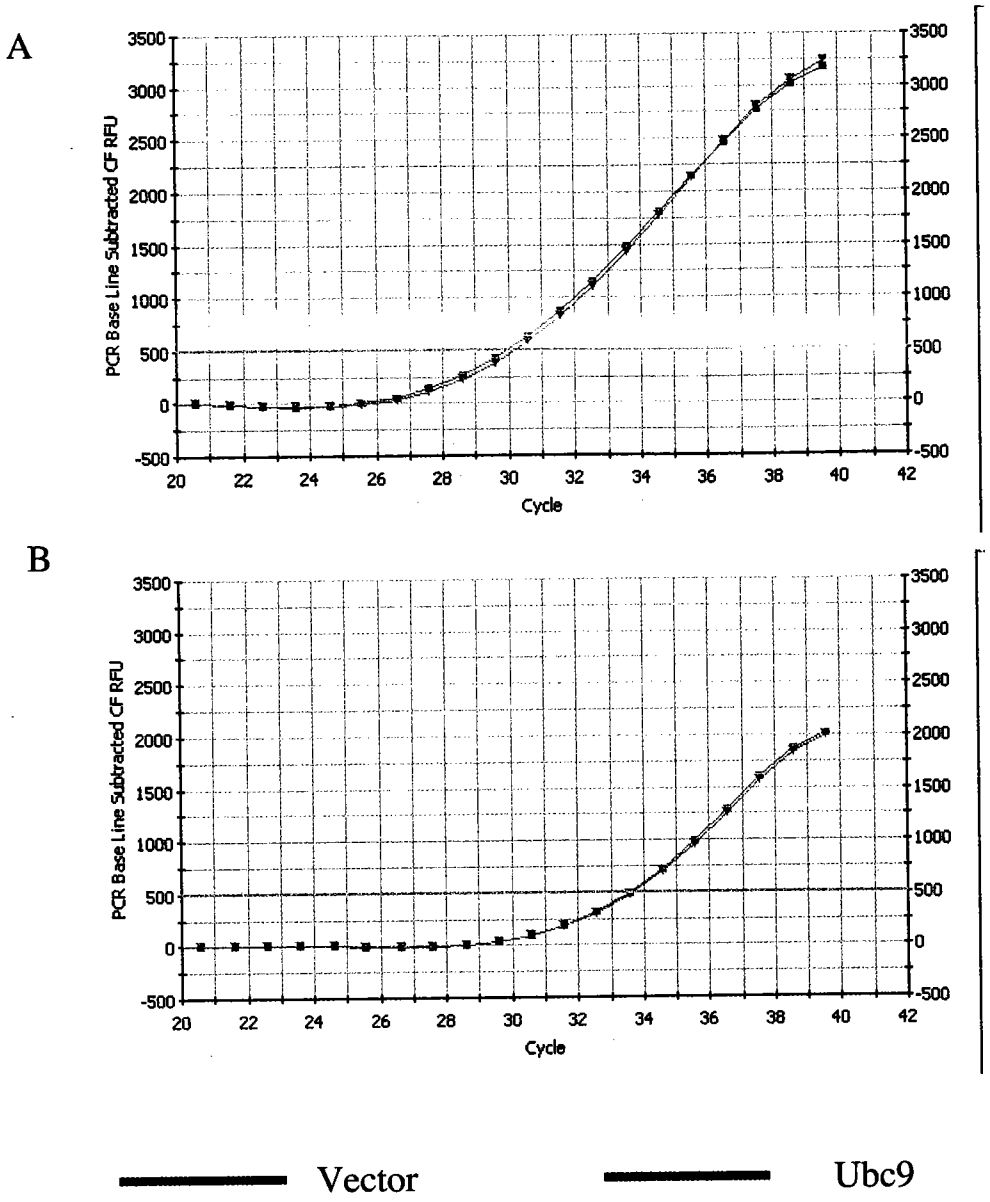


Fig 24. MECL-1mRNA levels are not affected by UBC9 overexpression. mRNA was extracted from COS-K^b cells transfected with Ubc9 or control vector and used in RT-real-time PCR. A: internal control HPRT. mRNA; B: MECL-1 mRNA. ***Note: the traces are overlayed.***

I then examined the effects of UBC9 overexpression on protein expression by western blotting. Endogenous TAP1 and TAPasin levels in COS cells were too low to be detected with the antibodies used, or possibly the African Green Monkey protein in COS cells fails to react with the anti-human antibodies that are available, and in the absence of IFN induction MECL-1, LMP2, and LMP7 are expressed at very low levels that were also not detectable. I therefore co-transfected plasmids expressing UBC9 and MECL-1, LMP-2, PA28α, or PA28β. In the presence of UBC9, there was significantly more protein detected of MECL-1, LMP-2, PA28α, and PA28β (Fig 25A-D).

Similarly, I co-transfected COS-K^b cells with tapasin. We found that UBC9 itself did not increase tapasin expression, but in the presence of co-transfected SUMO-2, UBC9-transfected cells expressed much higher levels of tapasin than did control vector-transfected cells (Fig 26). Very interestingly, the catalytically inactive mutant UBC9OH strongly increased tapasin levels when co-transfected with tapasin (Fig 27). It is not clear why UBC9OH by itself affected tapasin while the wild type UBC9 was only effective with SUMO2. Nevertheless, both of these findings suggest that UBC9 regulates tapasin protein levels, consistent with the immunostaining observations (Fig 23e)

UBC9 could increase protein levels without altering transcription either by increasing translation, or by reducing protein degradation. To examine these possibilities, COS cells were co-transfected with UBC9, and LMP2 or PA28β. Twenty-four hours after transfection, the cells were pulsed with ^{35}S-methionine for 20 minutes, and then chased for

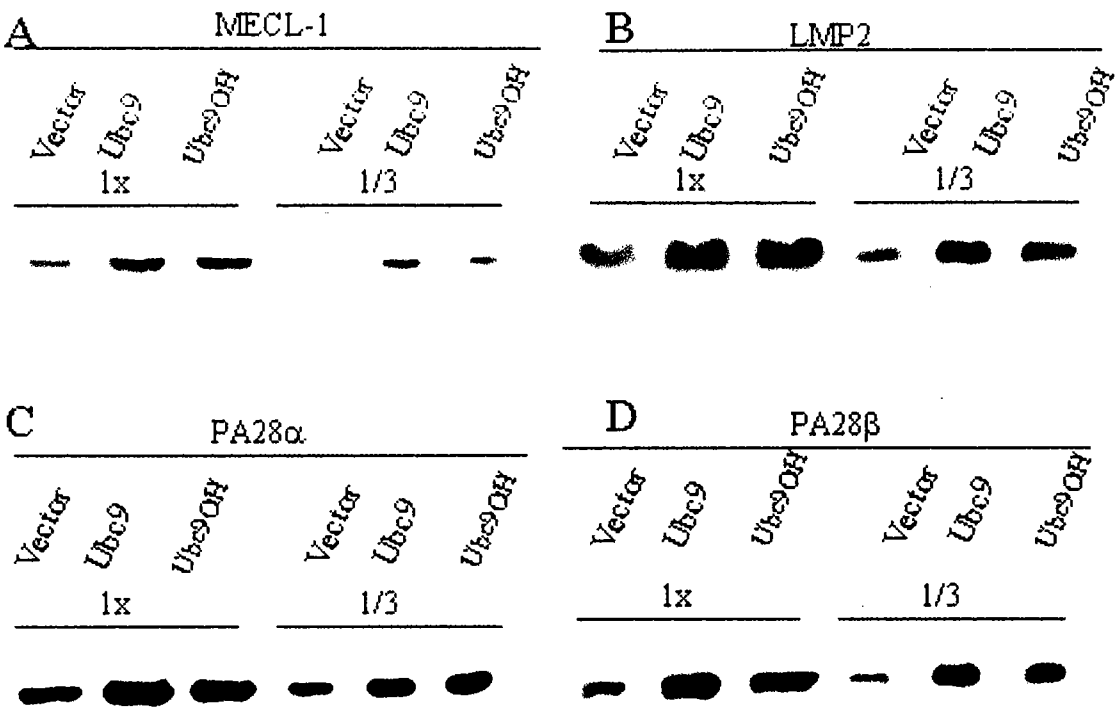


Fig 25. UBC9 increases levels of several proteins associated with antigen presentation. COS-Kb cells were co-transfected with MECL1 (A) / LMP2 (B) / PA28α (C) / PA28β (D) and control vector, Ubc9 or Ubc9OH. Twenty-four hours after transfection, the cells were lysed and a western blot was performed. Membranes were probed with anti-MECL1 (A), anti-LMP2 (B), anti-PA28α (C), and anti- PA28β (D) antibody respectively.

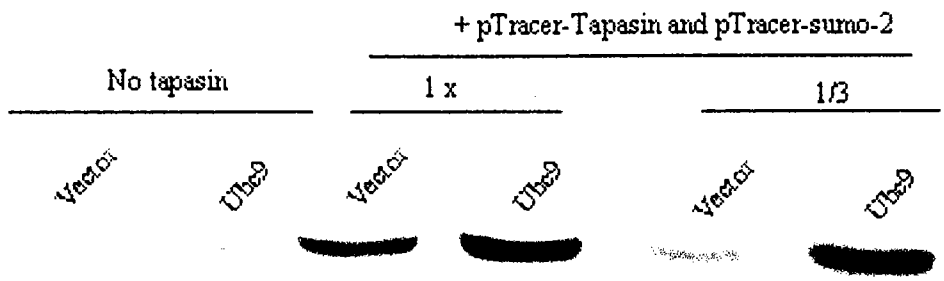


Fig 26. Ubc9 and sumo-2 overexpression increases tapasin protein level. COS-Kb cells were transfected with vector or Ubc9, or co-transfected with Vector or Ubc9, pTracer-CMV2-Tapasin, and pTracer-sumo-2. Twenty-four hours after transfection, the cells were lysed and a western blot was performed using anti-tapasin antibody. Note, without tapasin plasmid, tapasin protein can not be detected with antibody.

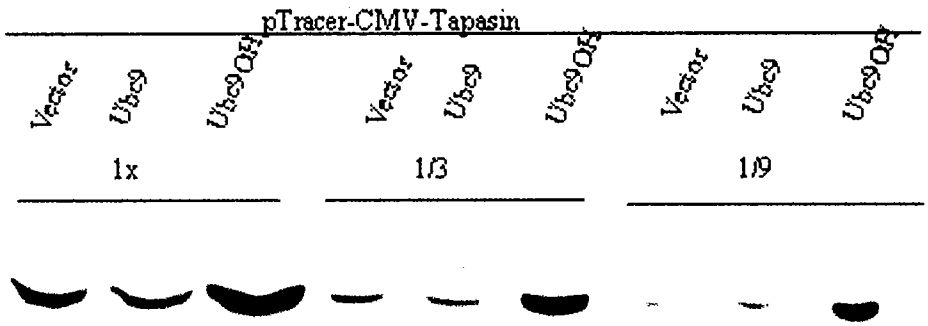


Fig 27. Ubc9OH overexpression increases tapasin levels. COS-Kb cells were co-transfected with tapasin and control vector, Ubc9 or Ubc9OH. Twenty-four hours after transfection, the cells were lysed and a western blot was performed using anti-tapasin antibody.

up to 3 hours (PA28β) or 7 hours (LMP). In the presence of UBC9, MECL-1 had significantly longer half-lives, showing that UBC9 inhibited their degradation (Fig 28). In contrast, UBC9 has no effect on the degradation rate of ovalbumin (see above and data not shown). Therefore UBC9 selectively post-translationally regulates the expression of several components of the MHC class I antigen presentation pathway.

3.5. siRNA-mediated elimination of UBC9 does not decrease MHC class I antigen presentation

Small interfering RNA (siRNA) has been used by many groups to eliminate or reduce specific gene expression (1, 203-206). To study the importance of UBC9 in vivo, we used siRNA to reduce UBC9 expression in COS-Kb cells. Twenty four hours after siRNA transfection, UBC9 levels were much lower than untransfected and control-transfected cells (Fig 29A), and three days after transfection UBC9 was almost completely undetectable (Fig 29B). When UBC9 siRNA-transfected cells were grown for more than 4 days, the cells were very sick, with most cells becoming round, and losing adherence to the plate (data not shown). When passed to another dish, the treated cell number did not increase (data not shown), suggesting that in mammalian cells, as in yeast (123), UBC9 is essential for cell viability.

The cytopathic effects of UBC9 siRNA treatment make it difficult to interpret any biological effects of UBC9 loss. With this caveat in mind, we studied the effects of eliminating UBC9 on MHC class I antigen presentation. COS-K^b cells were transfected with

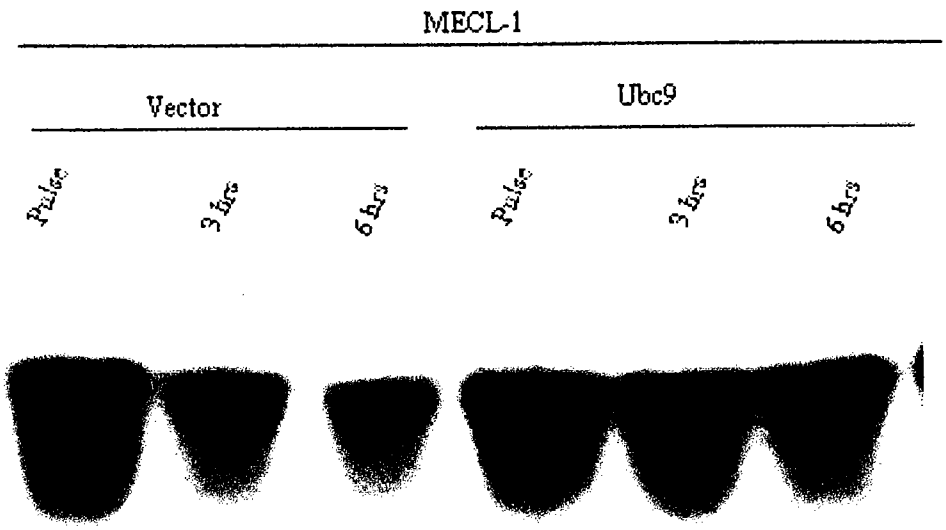


Fig 28. UBC9 overexpression stabilizes MECL-1. COS-K^b cells were co-transfected with plasmids expressing MECL-1 and empty vector or UBC9. Twenty-four hours after transfection, the cells were pulsed for 20 minutes and chased for various times, and immunoprecipitated using anti-MECL-1 antibody.

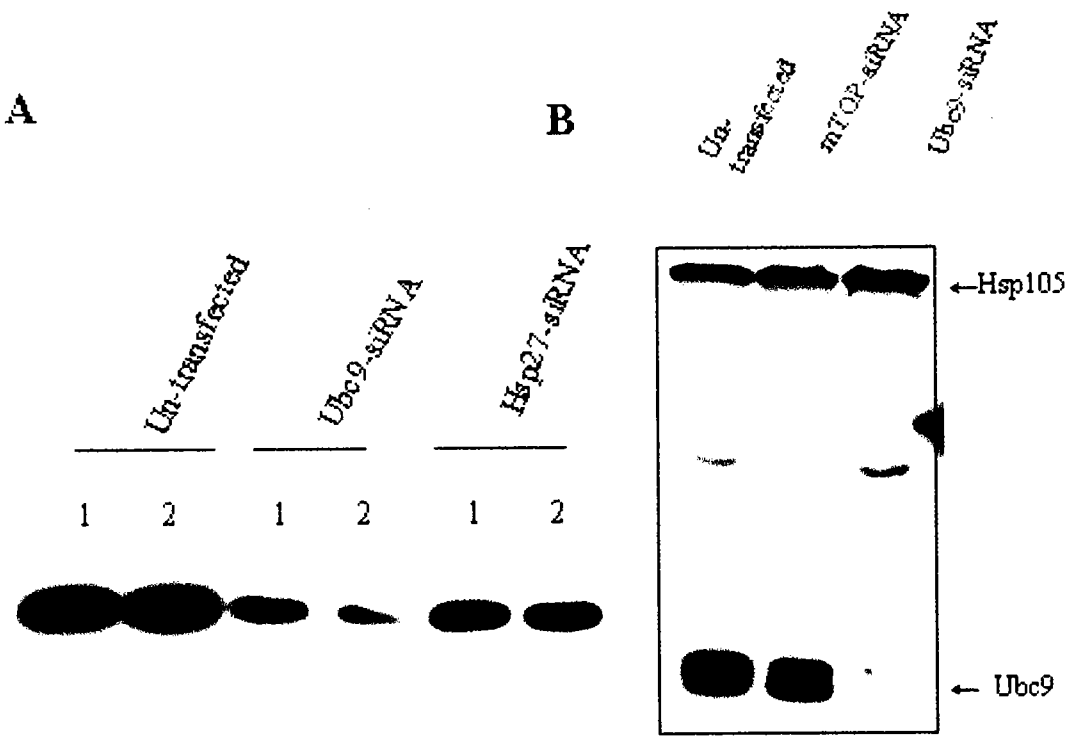


Fig 29. siRNA reduces Ubc9 levels in COS-Kb cells. COS-Kb cells were transfected with siRNA. One day (A) and three days (B) after transfection, the cells were lysed and western blots were performed using anti-UBC9 antibody.

UBC9 siRNA or control mTOP siRNA (1), and 3 days later, the cells were infected with recombinant vaccinia virus expressing full-length ovalbumin, ER-targeted SIINFEKL, or MSIINFEKL for 7 - 8 hours, and analyzed by flow cytometry (Fig 30). Although we expected that eliminating UBC9 would have the opposite effect to overexpression, surprisingly, UBC9 knockout increased the overall H-2K^b expression on the cell surface. UBC9 knockout also increased H-2K^b-SIINFEKL levels on cells infected with vaccinia virus expressing ER-targeted SIINFEKL and MSIINFEKL, but not on cells expressing full-length ovalbumin. This is in contrast to the results of UBC9 overexpression, in which UBC9 dramatically increased H-2K^b-SIINFEKL level in full-length ovalbumin transfected cells (Figure 4; Figure 5B). Nevertheless, in general, the findings that UBC9 inhibition increased H-2K^b-SIINFEKL level on ER-target SIINFEKL and MSIINFEKL-expressing cells contradict our prediction. Although we do not know the exact reason for this, one possibility is that UBC9 elimination stresses the cells, which may in turn up-regulate chaperones in the ER and enhance MHC class I assembly. Another possibility is that these results reflect an effect of UBC9 on the proteasome, as we concluded with overexpression studies. It is possible that, in the absence of UBC9, there will be less proteasome-generated peptides to compete with ES-SIINFEKL minigene and SIINFEKL minigene-generated authentic peptides, which in turn have more chance to bind to empty MHC class I molecules. However, we feel it is difficult to draw any firm conclusions given the cytopathic effects of eliminating UBC9.

3.6. Interferon treatment decreases UBC9 expression

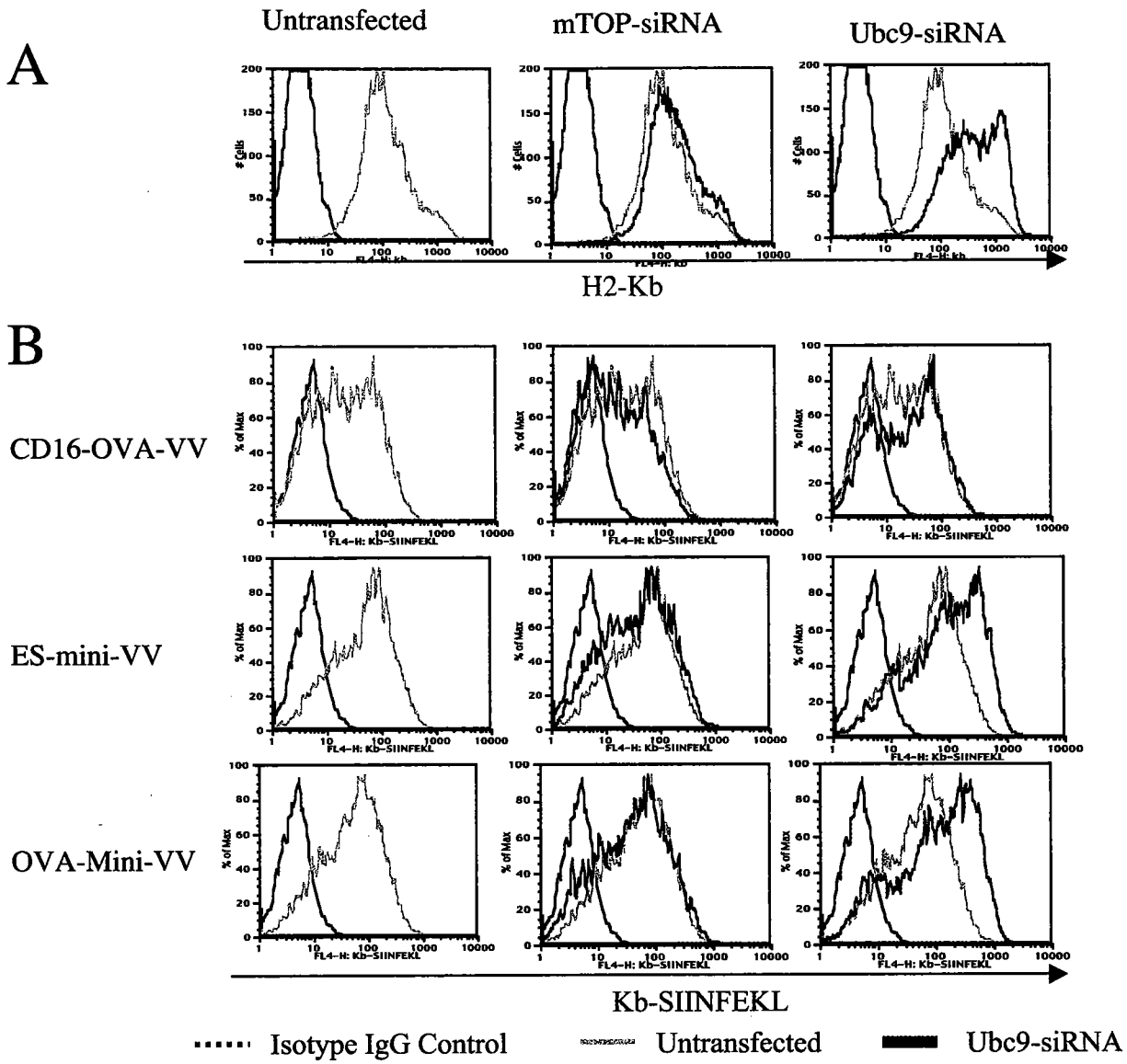


Fig 30. Ubc9 knockdown increases H-2Kb-SIINFEKL level from some SIINFEKL precursors. COS-Kb cells were transfected with control (mTOP) siRNA or Ubc9 siRNA. Four days after transfection, the cells were stained with Y3 antibody (A), or infected with recombinant vaccinia virus (moi=10) (B). Seven hours after infection, the cells were stained with 25.D1.16 antibody and analyzed by flow cytometry.

Both type I and type II interferon (IFN) have been shown to increase MHC class I antigen presentation (207). A gene chip study showed that treatment of cells with the type I INF-inducer poly I::C up-regulated UBC9 mRNA in dendritic cells (208). It seemed possible that one way in which IFN regulates MHC class I presentation is via UBC9. To test this, I treated COS-K^b cells with poly I::C, which induces type I IFN generation, or with human IFNγ (HuIFNγ). Both poly I::C and HuIFNγ increased MHC class I expression level (Fig 31A), confirming that the polyI::C and IFN were effective. However, these treatments decreased UBC9 expression (Fig 31B), suggesting that IFN does not increase MHC class I antigen presentation by increasing UBC9 expression.

To know whether IFN up-regulates UBC9 only in dendritic cells, I used mouse bone marrow-derived primary dendritic cells, and human monocyte-derived dendritic cells. These cells were treated with PolyI::C and IFNγ. Both treatments decreased UBC9 level on both kinds of dendritic cells (Fig 32),confirming that (in contrast to the gene chip findings) IFN does not up-regulate UBC9 expression.

3.7. SUMO-2 may be involved in UBC9 regulation of MHC class I antigen presentation.

UBC9 conjugates SUMO molecules, but not ubiquitin, to target proteins. In mammalian cells, there are at least 3 forms of SUMO molecules, SUMO-1 and two highly homologous SUMO molecules – SUMO-2 and SUMO-3. Although (at least in COS cells) there are more SUMO-2 and SUMO-3 molecules than SUMO-1 (172), the functions of

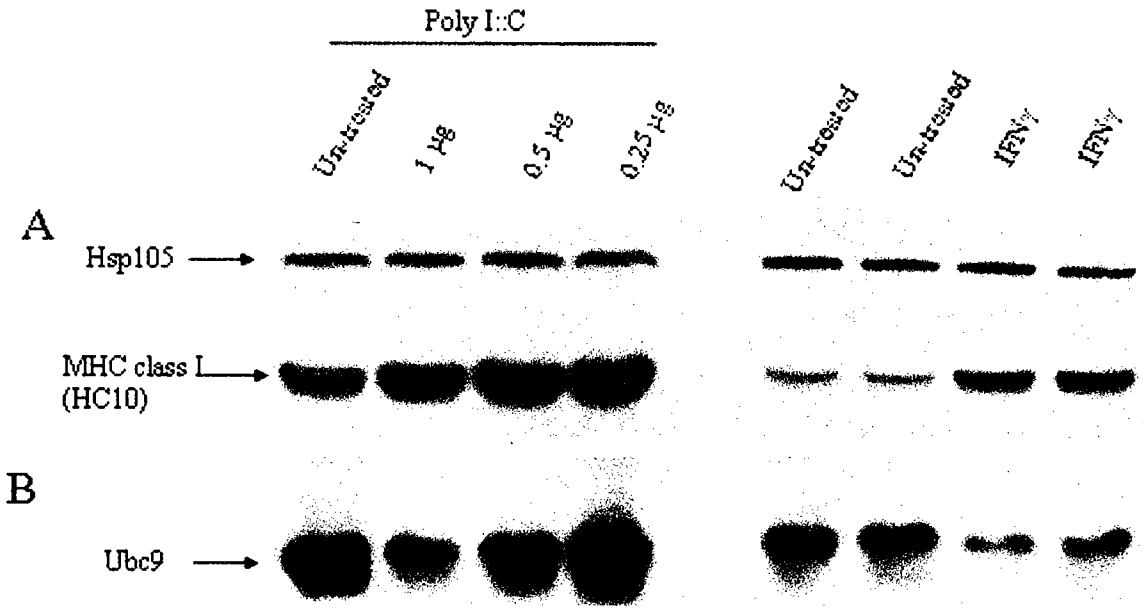


Fig 31. Interferon treatment decreases Ubc9 levels. COS-K^b cells were treated with Poly I::C or IFNγ for 24 hours. The cells were lysed and a western blot was performed with anti-Ubc9 antibody, MHC class I antibody HC10, and control antibody Hsp105.

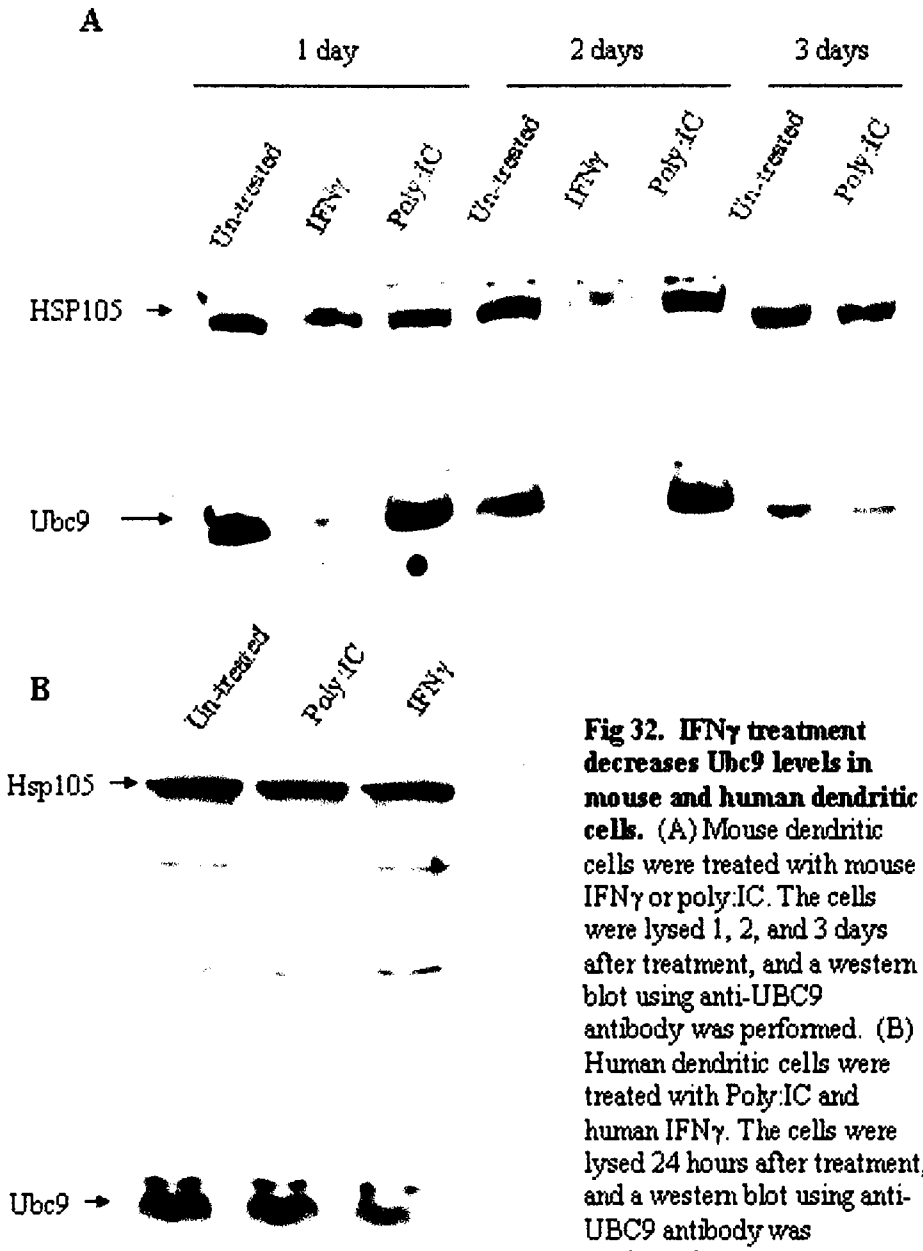


Fig 32. IFNγ treatment decreases Ubc9 levels in mouse and human dendritic cells. (A) Mouse dendritic cells were treated with mouse IFNγ or poly:IC. The cells were lysed 1, 2, and 3 days after treatment, and a western blot using anti-UBC9 antibody was performed. (B) Human dendritic cells were treated with Poly:IC and human IFNγ. The cells were lysed 24 hours after treatment, and a western blot using anti-UBC9 antibody was performed.

SUMO-2 and SUMO-3 in vivo are not known and no proteins modified by SUMO-2 or SUMO-3 molecules in vivo have been identified. Since UBC9 conjugates SUMO molecules to target proteins, we wanted to know which SUMO molecule(s) might be involved in UBC9's regulation of MHC class I antigen presentation. SUMO molecules are conjugated to their substrate lysine residue via their C-terminal gly-gly motif (209). To study this, I generated mutant SUMO-1, -2 and –3, in which their C-terminal Gly-Gly conjugating sequences have been mutated to ala-ala amino acids.. If a SUMO molecule is involved in UBC9 function on MHC class I antigen presentation, transfection of a wild type SUMO should have different effects on MHC class I antigen presentation then its mutant version; this should help distinguish specific from non-specific effects of the transfection.

The stably-transfected cell line 618-CD16-OVA (COS-Kb cells stably expressing full-length ovalbumin) was co-transfected with UBC9 together with either wild-type or mutant SUMO-1, -2, or -3. Twenty-six hours after transfection, the cells were analyzed by flow cytometry (Fig 33). H-2K^b-SIINFEKL expression levels showed no difference between cells transfected with wild-type and mutant SUMO-1 or SUMO-3 and UBC9. However, wild-type SUMO-2-transfected cells expressed higher levels of H-2K^b-SIINFEKL than did mutant SUMO-2-transfected cells when co-transfected with UBC9, suggesting the involvement of SUMO-2 in UBC9 enhancement of MHC class I antigen presentation. 618-CD16-OVA cells were then transfected with combinations of plasmids expressing UBC9 (wild-type and mutant) and SUMO-2 (wild-type and mutant). Thirty hours after transfection, the cells were analyzed by flow cytometry (Fig 34). SUMO-2 by itself, but not mutant SUMO-2 by itself, increased H-2K^b-SIINFEKL level, and UBC9OH by itself had

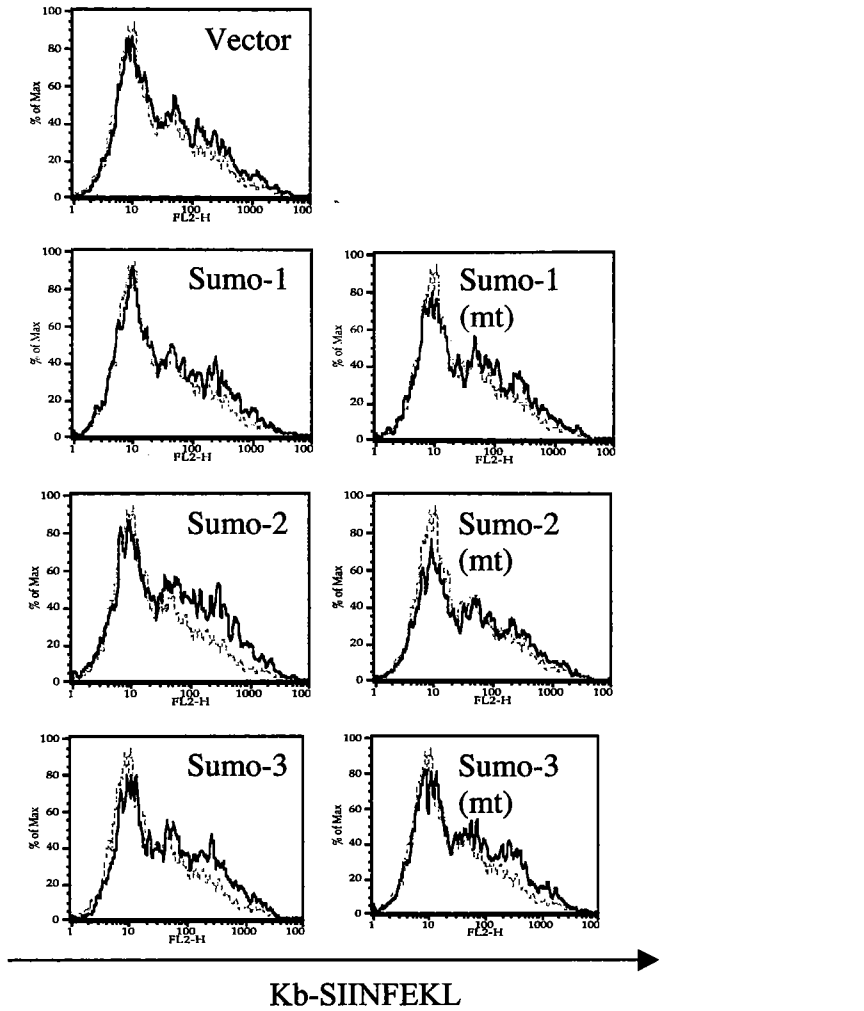


Fig 33. Sumo2 and mutant Sumo-2 behave differently in Ubc9 regulating MHC class I antigen presentation. COS-Kb cells stably expressing CD16-OVA were transfected with different plasmid combinations as indicated. Twenty-four hours after transfection, the cells were stained with 25.D1.16 antibody and GFP+ cell populations were analyzed by flow cytometry.

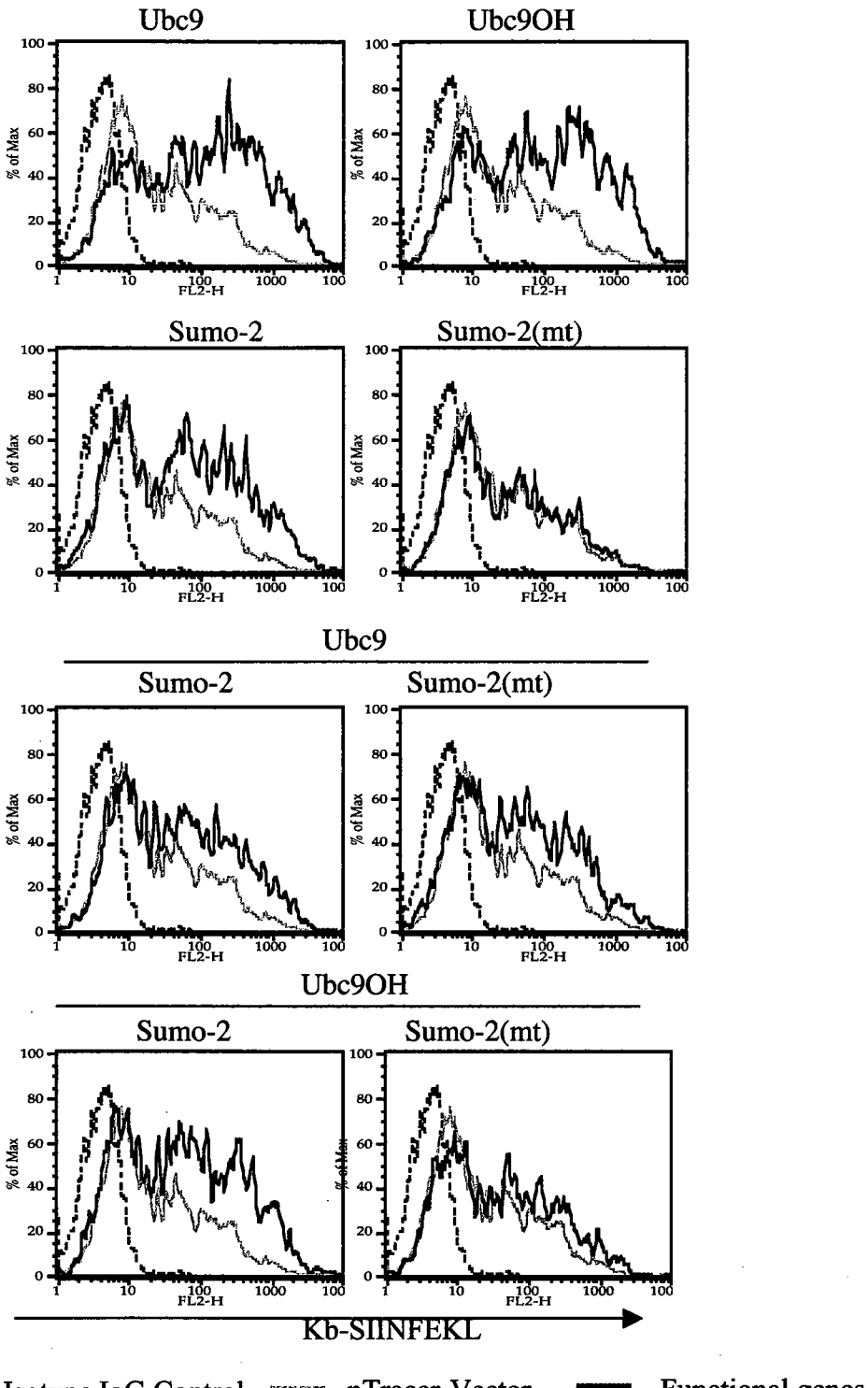


Fig 34. Sumo-2, but not mutant Sumo-2, over-expression increases Kb-SIINFEKL level. COS-Kb cells stably expressing CD16-OVA were transfected with plasmid combinations as indicated. Thirty hours after transfection, the cells were stained with 25.D1.16 antibody and GFP+ cell populations were analyzed by flow cytometry.

the same effect as UBC9 in regulating MHC class I antigen presentation, which is consistent with our early finding. However, although co-transfection of UBC9OH and SUMO-2 increased H-2K^b-SIINFEKL level on the cell surface, the co-transfection of UBC9OH with mutant SUMO-2 eliminated the effect of UBC9OH itself, which was in contrast with the results of co-transfection with UBC9 and mutant SUMO-2. In summary, mutant SUMO-2 did not increase class I expression by itself and blocked the increase induced by mutant UBC-9 (see table 3).

Table 3: Summary of the effects of wild-type and mutant UBC9 and SUMO molecules on antigen presentation.

UBC9	SUMO	Effect on Antigen Presentation
UBC9	-	+++
UBC9OH	-	+++
-	SUMO1	-
-	SUMO1mut	-
-	SUMO3	-
-	SUMO3mut	-
-	SUMO2	+++
-	SUMO2mut	-
UBC9	SUMO-1	++
UBC9	SUMO1mut	++
UBC9	SUMO3	++
UBC9	SUMO3mut	++
UBC9	SUMO2	++
UBC9	SUMO2mut	+
UBC9OH	SUMO2	++
UBC9OH	SUMO2mut	-

I also performed antigen loading experiments to confirm that SUMO-2 plays a role in MHC class I antigen presentation. COS-K^b cells were co-transfected with different UBC9, UBC9OH, SUMO-2 and mutant SUMO-2 plasmid combinations. Twenty-eight hours after transfection, the cells were loaded with ovalbumin protein and incubatedfor 2-2.5 hours. The cells were analyzed by flow cytomety (Fig 35). The results were very similar to the transient transfection experiments shown above. When mutant UBC9 and mutant SUMO-2

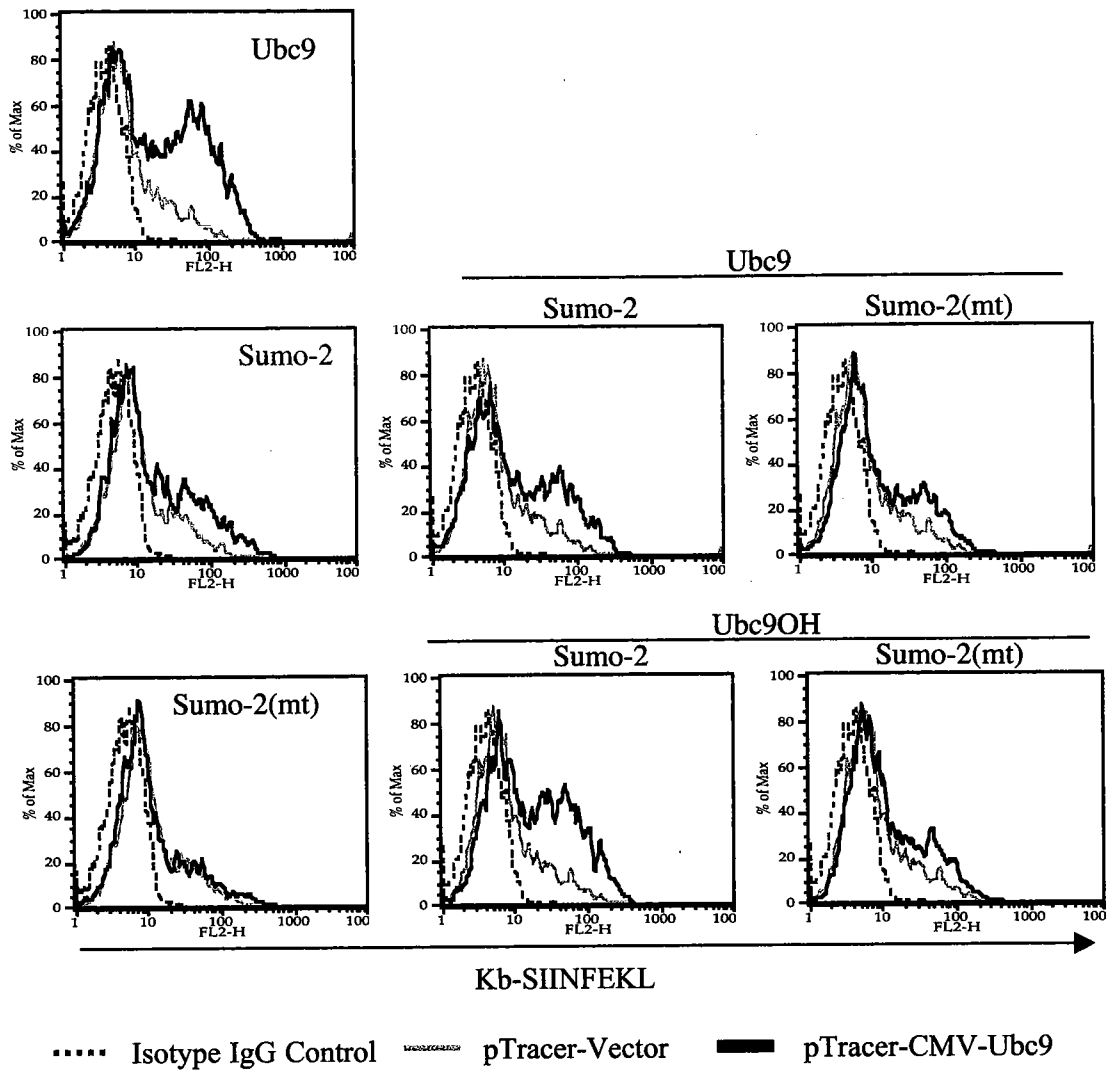


Fig 35. Sumo-2 enhances MHC class I antigen presentation. COS-Kb cells were transfected with different plasmid combinations as indicated. Twenty-eight hours after transfection, the cells were loaded with ovalbumin and incubated for 2 hours, stained with 25.D1.16 antibody, and GFP+ cell populations were analyzed by flow cytometry.

were used in combination, the enhancing effect of UBC9 was almost totally lost, suggesting the interaction of UBC9 and SUMO-2 in MHC class I antigen presentation.

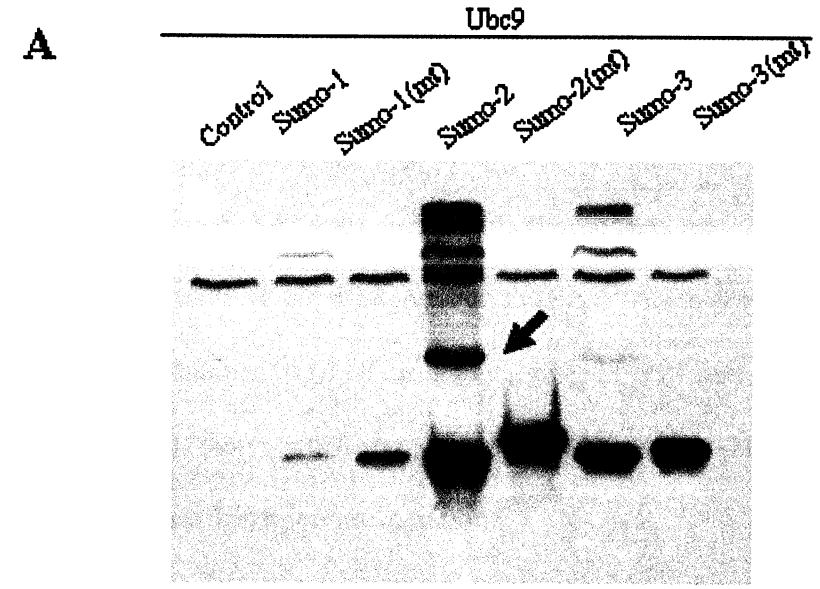
CHAPTER IV

HSP27 AFFECTS MHC CLASS I ANTIGEN PRESENTATION

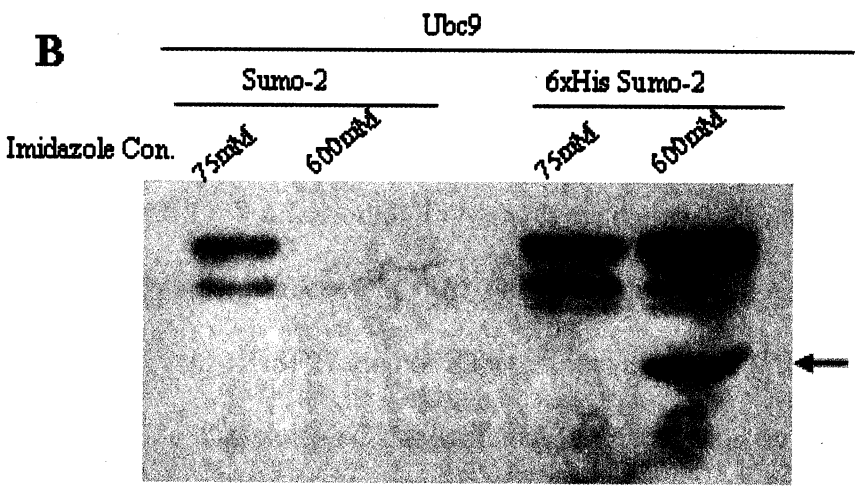
4.1 HSP27 overexpression reduces H-2K^b-SIINFEKL levels

To study how UBC9 regulates MHC class I antigen presentation, COS-K^b cells were cotransfected with UBC9 and 6xHis/Xpress tagged SUMO-2 or mutant SUMO-2. Twenty-eight hours after transfection, the cells were lyzed and the lysates were subjected to immunoprecipitation with NTA-Zn^{2+} bead. Western blot results showed a 38 kDa protein was recognized by anti-Xpress antibody in SUMO-2-transfected, but not mutant SUMO-2-transfected, cell lysates. Therefore, this 38kDa band represented a SUMO-2 conjugated protein (Fig 36A). HSP27 has been reported to interact with UBC9 in vivo (210). When the membrane was probed with rabbit-anti-mouse HSP27 antibody, a band of 38 kDa was detected (Fig 36B). However, further examination determined that the 38 kDa band was actually a UBC9-SUMO-2 complex that coincidentally co-migrated with HSP27 (data not shown).

Even though the original observation focusing my attention on HSP27 turned out to be an artifact, I had performed experiments with HSP27 before this was resolved and found that altering HSP27 expression did in fact affect antigen presentation. Hypothesizing that UBC9 affects MHC class I antigen presentation by regulating HSP27, I cloned HSP27 by



WB: Probed with anti-HA . All SUMOs are HA tagged.



WB: Probed with anti-HSP27 .

Fig 36. SUMO-2 forms a conjugate to a protein that co-migrates with HSP27. A: COS-K^b cells were co-transfected with plasmids expressing UBC9 and wild-type or mutant HA-tagged SUMO molecules. After **28 hours** the cells were lysed and a western blot was performed using anti-HA antibody. The arrow indicates **specific band conjugated by wild type but not mutant SUMO-2** B: COS-K^b cells were transfected with plasmids expressing UBC9 and SUMO-2 or 6xHis tagged SUMO-2. After **28 hours** the cells were lysed and the lysates were applied to a NTA-Ni column. After elution with Ni^{2+}, western blots were pferformed on the eluates using anti-HSP27 antibody. The arrow indicates **a band recognized by anti-HSP27 antibody.**

RT-PCR and co-transfected E36-K^b cells with HSP27 and various SIINFEKL precursors. Nineteen hours after transfection, the cells were analyzed by flow cytometry (Fig 37). HSP27 overexpression inhibited MHC class I antigen presentation of SIINFEKL derived from ovalbumin targeted to the ER, the cytosol, or lysosomes, from SIINFEKL targeted to the ER, and from MSIINFEKL itself. Although only small effects were seen in COS-K^b cells co-transfected with HSP27 and ER-targeted ovalbumin (Fig 38A), in protein loading experiments with full-length ovalbumin COS-K^b cells expressing HSP27 had significantly lower levels of H-2K^b-SIINFEKL than control-transfected cells (Fig 38B). Thus HSP27 overexpression reduces MHC class I antigen presentation.

As with UBC9, HSP27 could affect any, or many, of the components of the antigen presentation pathway. To know whether HSP27 inhibits overall protein synthesis or protein trafficking, E36-K^b cells were co-transfected with pTracer-HA-HSP27 (encoding HA-tagged HSP27) and pTracer-CD16-OVA (encoding full-length ovalbumin), pTracer-CMV-H1 (encoding influenza virus HA), or pTracer-CMV-A*0302 (encoding HLA-A3) and analyzed by flow cytometry (Fig 40). HSP27 did not affect protein synthesis (Figure 39) but inhibited H-2K^b-SIINFEKL expression, and also inhibited HA expression on the cell surface (one of two times), but did not affect HLA-A3 expression. This result suggests that HSP27 affects the transport of some, but not all, proteins to the cell surface. So, this effect is not specific for MHC class I but instead affects several cell-surface glycoproteins.

4.2. Mutation of potential SUMO conjugation and phosphorylation sites does not affect HSP27's effect on MHC class I antigen presentation

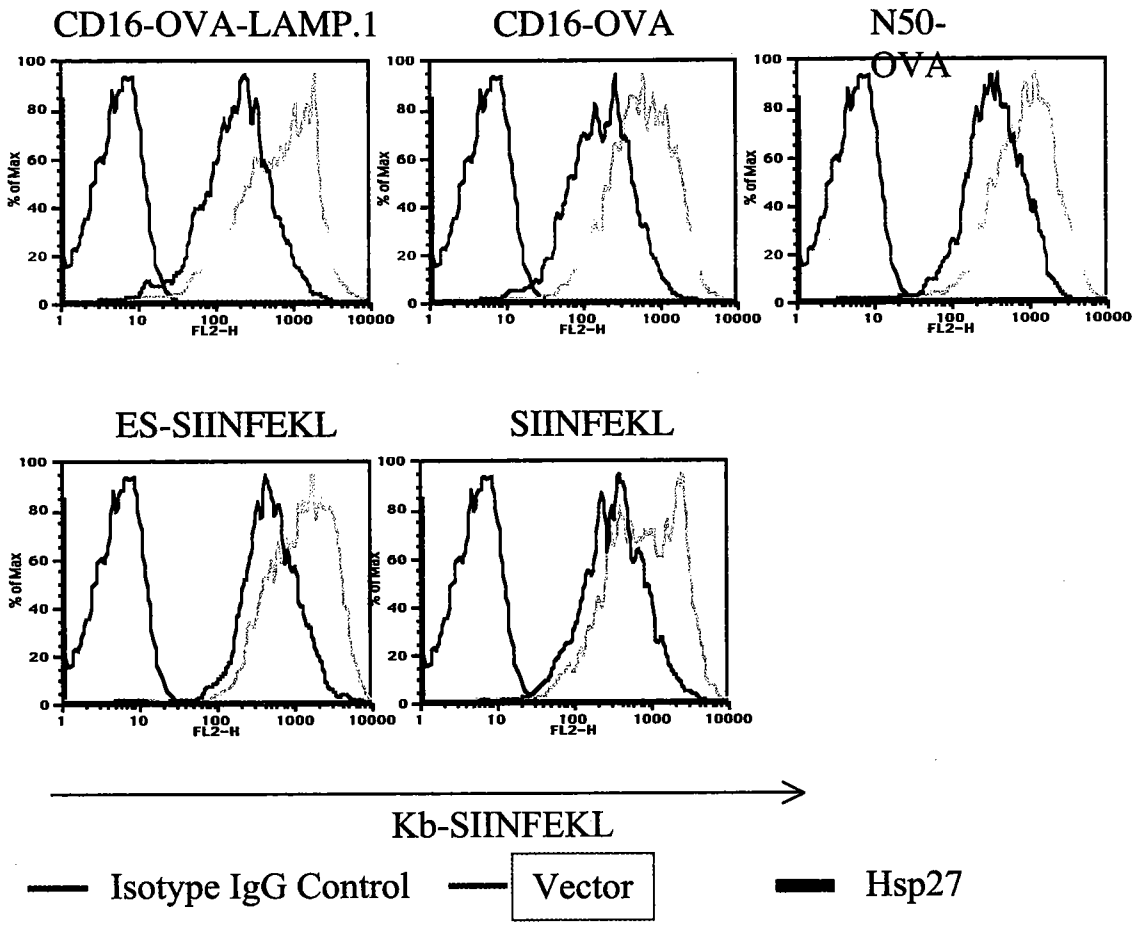


Fig 37. Hsp27 overexpression down-regulates H-2Kb-SIINFEKL levels. E36-Kb cells were co-transfected with different plasmid combinations as indicated. Eighteen hours after transfection, the cells were stained with 25.D1.16 antibody and GFP+ cell populations were analyzed by flow cytometry.

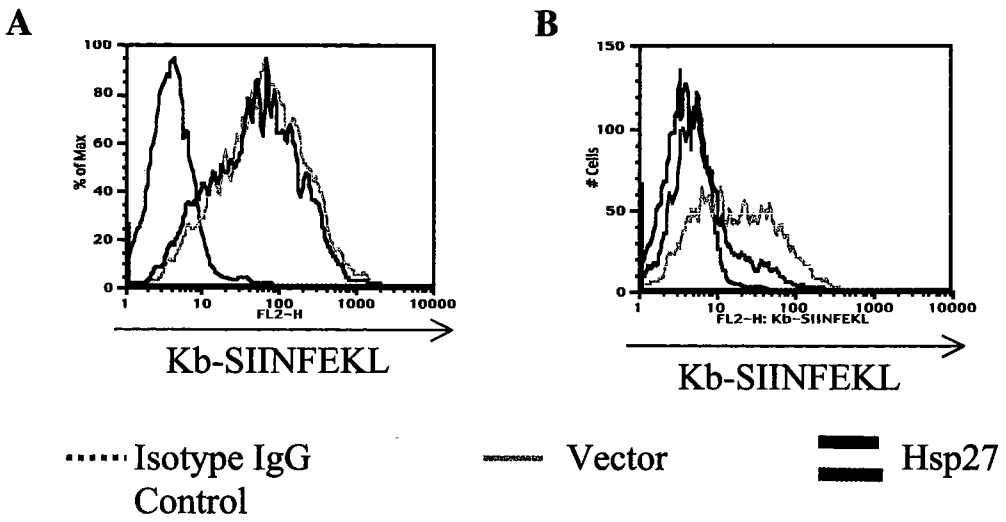


Fig 38. Hsp27 overexpression decreases H-2K^b-SIINFEKL generation from ovalbumin protein. A, COS-K^b cells were co-transfected with plasmids expressing ovalbumin and control vector or pTracer-CMV-Hsp27. Twenty-four hours after transfection, the cells were stained with 25.D1.16 antibody and GFP +ve cells were analyzed by flow cytometry. B. COS-K^b cells were transfected with control vector or pTracer-CMV-Hsp27. Twenty-four hours after transfection, the cells were loaded with ovalbumin by osmotic lysis of pinosomes. After 1 hour incubation at 37 °C, the cells were stained with 25.D1.16 antibody and GFP +ve cells were analyzed by flow cytometry.

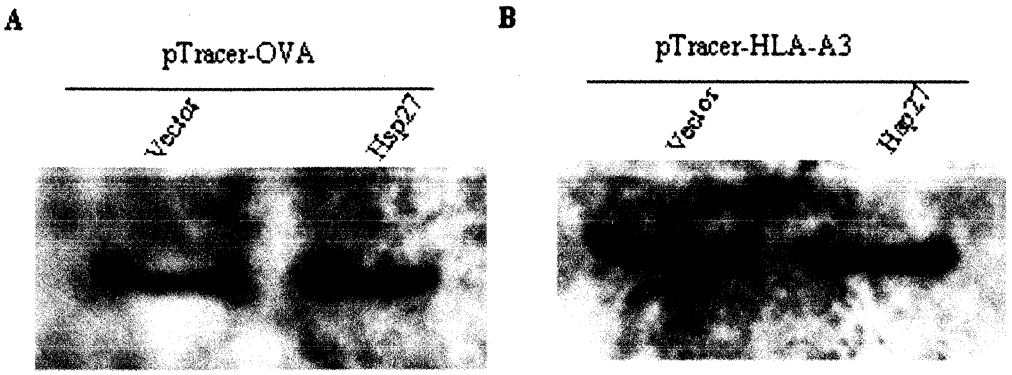


Fig 39. Hsp27 does not increase protein synthesis. COS-Kb cells were co-transfected with pTracer-OVA and control vector or Hsp27 (A), or co-transfected with pTracer-HLA-A3 and control vector or Hsp27. Twenty-four hours after transfection, the cells were lysed and western blot s were performed. Membranes were probed with anti-OVA (A) or anti-A3 (HCA2) antibody respectively

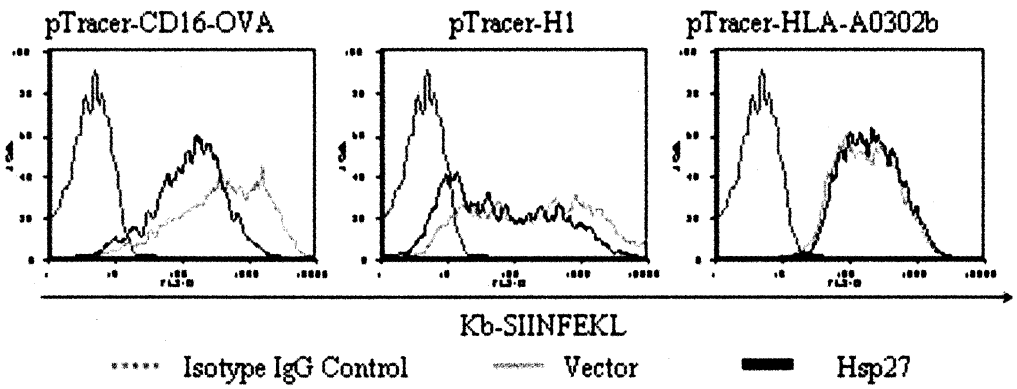


Fig 40. Hsp27 affects SIINFEKL-H-2K^b and hemagglutinin, but not HLA-A3, expression on the cell surface. E36-Kb cells were co-transfected with Hsp27 and pTracer-CD16-OVA (A), pTracer—H1 (B) or pTracer-HLA-A0302b (C). Eighteen hours after transfection, the cells were stained with 25.D1.16 (A), H36.4.2 (B), or GAP-A3 (C), and GFP +ve cells were analyzed by flow cytometry.

We initially hypothesized that the effect of UBC9 was related to its association with HSP27. Since UBC9 conjugates SUMO molecules to lysines on target protein, I mutated all lysines in HSP27 individually to arginines. E36-K^b cells were co-transfected with these mutant HSP27 and pTracer-CD16-OVA. I found that overexpression of all mutants of HSP27 inhibited MHC class I antigen presentation (Fig 41), suggesting HSP27 does not regulate MHC class I antigen presentation via its interaction with UBC9. Consistent with this, when COS-Kb cells were co-transfected with UBC9 and HSP27, HSP27 was not found to be modified by any SUMO molecule (data not shown).

HSP27 is a phosphoprotein. I mutated the three phosphorylation sites individually from serines to alanines. E36-K^b cells were co-transfected with these mutant genes and pTracer-CD16-OVA, and analyzed by flow cytometry. No mutation was found to relieve the inhibitory effect of HSP27 on MHC class I antigen presentation (Fig 42), implying that phosphorylation is not involved in HSP27 function in MHC class I antigen presentation. However, again, since I did not make double or triple mutations, I can not rule out the possibility that kinases can use multiple phosphorylation sites to modify the protein, which in turn affects MHC class I antigen presentation.

4.3. HSP27 knockout decreases MHC class I antigen presentation

To know how important a role HSP27 plays in MHC class I antigen presentation in vivo, siRNA was used to reduce HSP27 expression. Four days after siRNA transfection of COS-K^b cells, HSP27 expression was almost completely eliminated (Fig 43). As with UBC9 knockout cells, the cells lacking HSP27 were very sick and stopped growing, which

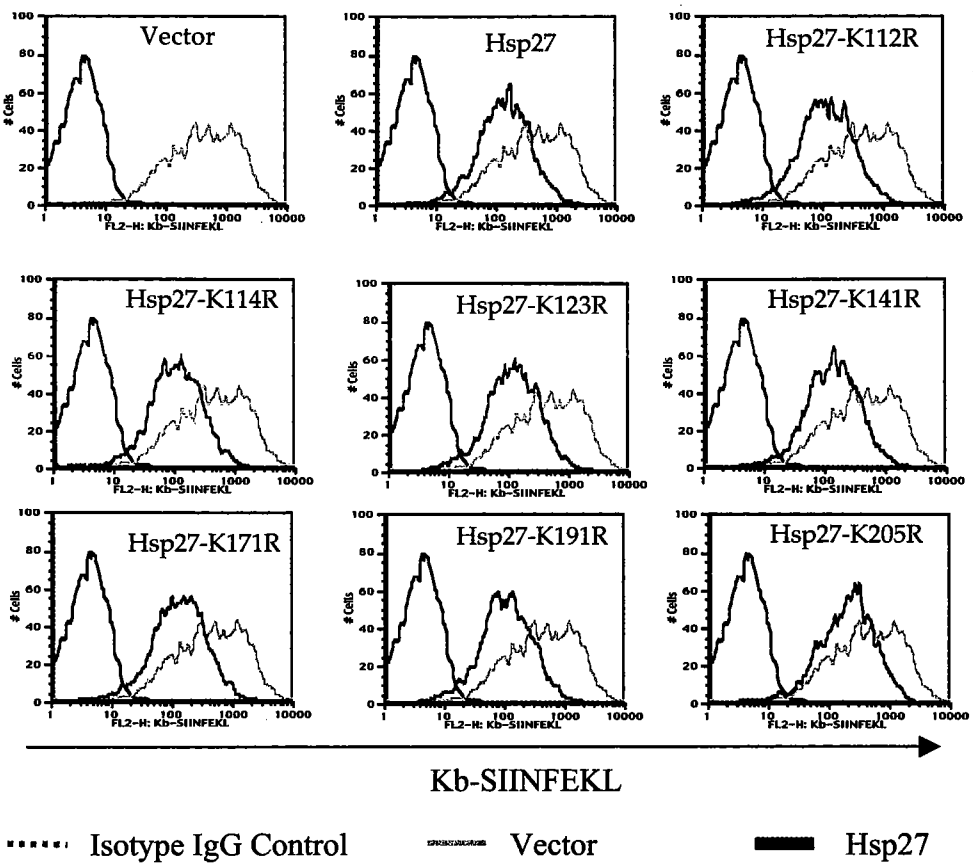


Fig 41. Mutation of potential Sumo conjugation sites does not affect Hsp27's inhibition of MHC class I antigen presentation. E36-Kb cells were co-transfected with pTracer-CMV-CD16-OVA and various Hsp27 mutants as described in the Materials and Methods. 18.5 hours after transfection, the cells were stained with 25.D1.16 antibody and GFP +ve cells were analyzed by flow cytometry.

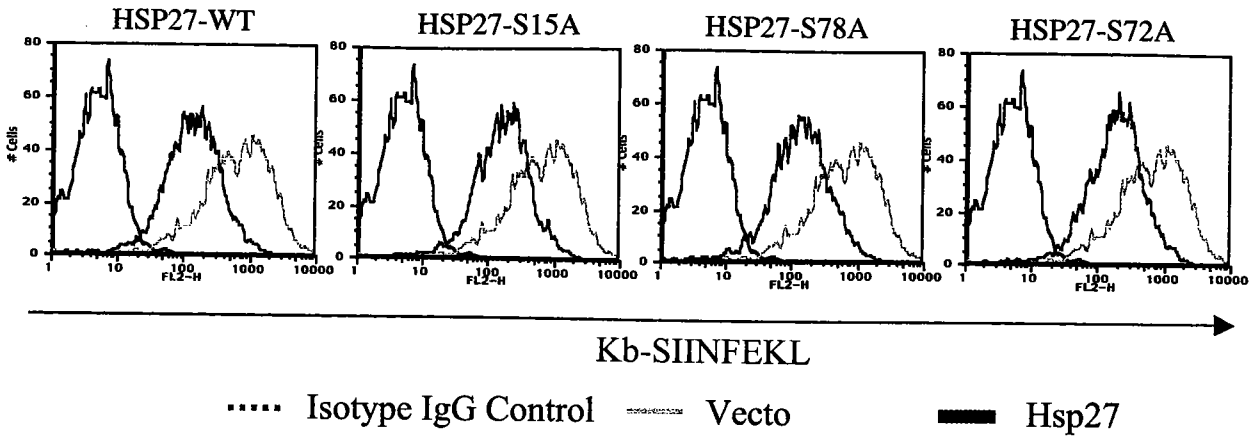


Fig42. Mutation of potential phosphorylation sites does not affect Hsp27's inhibition of MHC class I antigen presentation. E36-K^b cells were co-transfected with pTracer-CMV-CD16-OVA and various Hsp27 mutants as described in the Materials and Methods. Fourteen hours after transfection, the cells were stained with 25.D1.16 antibody and GFP+ cell populations were analyzed by flow cytometry.

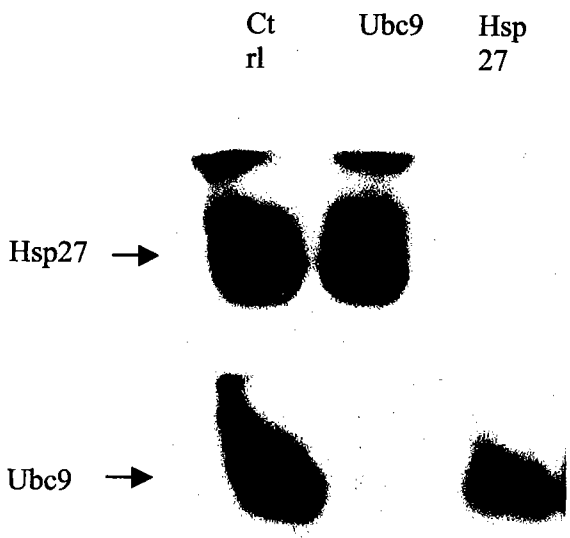


Fig 43. siRNA reduces levels of Ubc9 and Hsp27. COS-K^b cells were transfected with Ubc9-siRNA or Hsp27-siRNA. Four days after transfection, the cells were lysed and a western blots was performed using anti-UBC9 and anti-HSP27 antibodies.

is consistent with published data (211), suggesting that HSP27 may be essential for cell viability. When HSP27-knockout COS-K^b cells were transfected with several SIINFEKL precursors, H-$2K^b$-SIINFEKL levels on HSP27 knockout cells were always lower than on untransfected cells or control siRNA-transfected cells (Fig 44A): indeed the overall MHC class I level on HSP27 knockout cells was lower than on un-transfected cells and control siRNA transfected cells (Fig 44B). Similarly, when COS-7 cells were co-transfected with HSP27 and H-$2K^b$ or H-$2D^b$, lower MHC class I expression level on HSP27-transfected cells was observed than in control transfected cells (Fig 45). Interestingly, elimination of HSP27 had no effect on HLA-A3 expression, which is consistent with HSP27 overexpression experiments. The lack of an effect on HLA-A3 may reflect a difference in peptide supply that is dependent on the particular peptide motif bound by this class I allele (which is somewhat unusual in its preference for binding peptides with basic C-termini). However, in light of the effect of HSP27 on influenza hemagglutinin, it seems more likely that the effect of HSP27 is on glycoprotein transport or degradation in general, and for an as-yet unknown reason HLA-A3 is less affected by this than are H-$2K^b$ and H-$2D^b$.

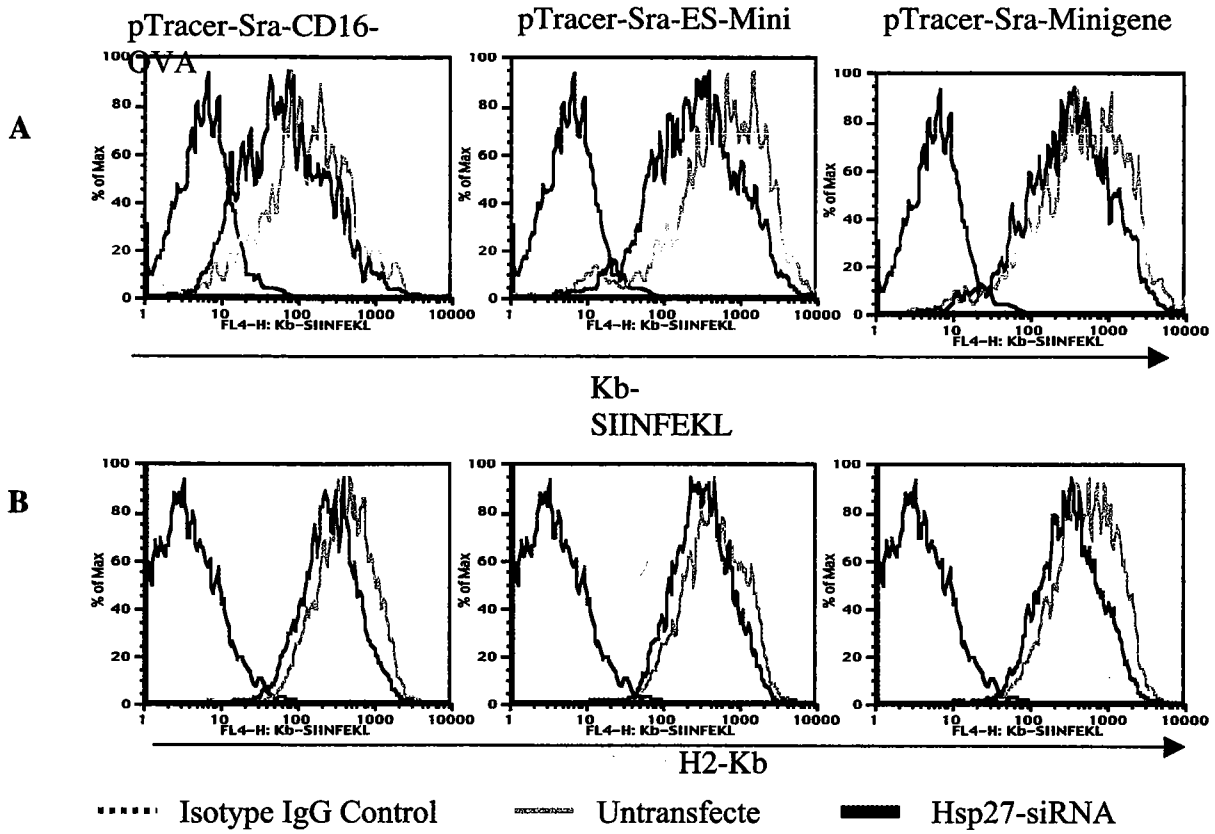


Fig 44. Hsp27 knockdown decreases H-2Kb-SIINFEKL levels on cells transfected with various ovalbumin genes. COS-Kb cells were transfected with control (mTOP) siRNA or Hsp27-siRNA. Three days after transfection, the cells were transfected with pTracer-Srα-CD16-OVA (expressing full-length ovalbumin targeted to the ER), pTracer-Srα-ES-minigene (expressing SIINFEKL targeted to the ER by a signal sequence), or pTracer-Srα-minigene (expressing MSIINFEKL in the cytosol). Twenty-two hours after transfection, the cells are stained with Y3 antibody (A), or 25.D1.16 antibody (B) and GFP+ cell populations were analyzed by flow cytometry.

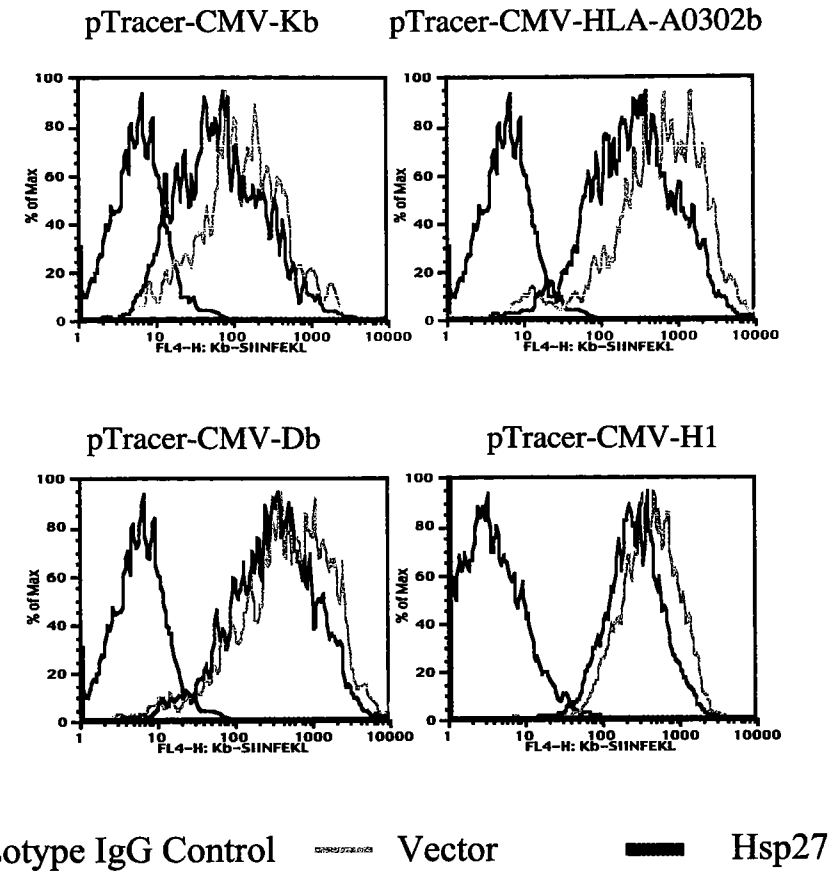


Fig 45. Hsp27 overexpression decreases levels of some cell-surface glycoproteins. COS-7 cells were co-transfected with plasmids expressing Hsp27 and H-2K^b, HLA-A*0302, H-2D^b, or influenza hemagglutinin (H1). Forty-eight hours after transfection, the cells were stained with appropriate antibodies and GFP+ cell populations were analyzed by flow cytometry.

CHAPTER V: DISCUSSION

5.1. UBC9 overexpression increases antigen presentation

In our studies of the effects of ubiquitin-conjugating enzymes on protein degradation and MHC class I antigen presentation, we originally used UBC9 as a control, since UBC9 conjugates SUMO, rather than ubiquitin, to target proteins (131, 132), and SUMO conjugation does not generally lead to protein degradation (149, 150). Unexpectedly, UBC9 overexpression significantly enhanced antigen presentation. Co-transfection of ovalbumin and UBC9 increased cell surface H-2K^b-SIINFEKL levels about 6- to 10-fold, compared to cells co-transfected with control vector and ovalbumin (Fig 1). The effect was not due to an increase in H-2K^b protein synthesis (Fig 1D and 1E), suggesting that UBC9 increased peptide supply to MHC class I molecules.

UBC9 was first identified as a nuclear protein in yeast (123), and has been reported to associate with many nuclear factors (126, 149, 150). It seemed possible that UBC9 might increase synthesis of the transfected antigens, thus indirectly increasing peptide supply as the proteins were degraded. However, immunoprecipitation of full-length and cytosolic ovalbumin from transfected cells did not show any difference in protein levels in the presence of UBC9 (Fig 1D). To further confirm that UBC9 does not up-regulate MHC class I antigen presentation by increasing protein synthesis, I bypassed protein synthesis by directly loading ovalbumin into the cytosol of cells by osmotic lysis of pinosomes. Even under these conditions UBC9-transfected cells expressed higher levels of H-2K^b-SIINFEKL

than control-transfected cells (Fig 6), confirming that the effects of UBC9 on antigen presentation are not mediated by increasing the synthesis of antigen.

Although most researchers have found that UBC9 is not involved in protein degradation, a few reports have demonstrated that UBC9 can lead to protein degradation (123, 127, 128). Enhancing the degradation of an antigen can lead to generation of a presented peptide (16). However, UBC9 overexpression did not accelerate ovalbumin degradation (Fig 8).

Many antigenic peptides are destroyed before they are presented by peptidases in the cytosol and ER. It was possible that UBC9 was enhancing presentation by reducing the degradation of these antigenic peptides. In collaboration with Dr. Tomo Saric in Dr. A.L. Goldberg's lab (Harvard Medical School, Boston, MA) I examined the stability of peptides in UBC9-overexpressing cells. UBC9 overexpression did not protect peptides from degradation in cytosolic extracts (Fig 21), or in intact cells (Fig 22). UBC9 therefore does not regulate MHC class I antigen presentation by protecting peptides from degradation.

5.1.1. UBC9 affects multiple steps in MHC class I antigen presentation

MHC class I antigen presentation is a multi-step pathway. In the cytosol, proteins are degraded by proteasomes, generating oligopeptides. These peptides may be further trimmed by aminopeptidases and transported into the endoplasmic reticulum lumen by the transporter associated with antigen processing (TAP) complex. Trimming of N-extended precursor peptides also occurs in the ER. Once generated, 8-10-residue long peptides bind

to empty MHC class I heavy chain/ β2-microglobulin complexes in the ER with the help of tapasin. Chaperones, including GRP78, calnexin, calreticulin, and ERp57, assist MHC class I assembly in the ER. I sought to identify the component(s) of the antigen presentation pathway that was affected by UBC9 overexpression.

To help localize the effect(s) of UBC9 on antigen processing, I hypertonically loaded cells with different precursors of SIINFEKL: SIINFEKL extended at the N-terminus (LEQLESIINFEKL) or at the C-terminus (SIINFEKLT), or SIINFEKL itself. H-2K^b-SIINFEKL levels on UBC9-transfected cells were always higher than control vector-transfected cells, no matter which precursor was used. This was unexpected, since these precursors have different requirements for proteolytic processing for the generation of SIINFEKL.

In the absence of UBC9, SIINFEKL antigen presentation was very inefficient after hypertonic loading of the C-terminal extended peptide SIINFEKLT, while UBC9 overexpression increased H-2K^b-SIINFEKL levels from this precursor at least tenfold. Similarly, when COS-K^b cells were co-transfected with plasmids expressing UBC9 and C-terminal extended SIINFEKL, H-2K^b-SIINFEKL levels on UBC9 transfected cells were much higher than on control vector transfected cells. In fact, UBC9 overexpression stimulated the greatest increase in presentation for the C-terminally extended peptides relative to all other constructs, suggesting that it was affecting the C-terminal trimming step (in addition to its other effects). The cleavage that generates the C-terminus of a presented peptide is absolutely dependent on proteasomes (47), suggesting that UBC9 is affecting

proteasome-mediated degradation. I also examined the unlikely possibility that UBC9 might promote protein degradation by a pathway other than the conventional ubiquitin-proteasome pathway, that might more efficiently generate peptides suitable for MHC class I presentation. However, the proteasome inhibitor MG132 completely inhibited H-2K^b-SIINFEKL expression on cells co-transfected with UBC9 and hypertonically loaded with ovalbumin (Fig 9); this demonstrated that proteasomes are required for ovalbumin degradation even when UBC9 is overexpressed.

On the other hand, the increase in presentation with LEQLESIINFEKL, which can bypass the proteasome and be trimmed by aminoeptidases, suggested that UBC9 might also affect aminopeptidases and/or some other step downstream of the proteasome. This seemed particularly plausible in light of a report that BH (an aminopeptidase that has been associated with antigen presentation (49)) and that has been reported to associate with UBC9 (202), although, I have been unable to detect any association between UBC9 and BH). Ubc9 co-transfection increased the expression of BH in cells. Moreover, transfecting BH itself into cells augmented antigen presentation (Figure 20). Therefore, one of the ways that Ubc9 may augment antigen presentation may be to increase BH and thereby the trimming of N-extended precursor peptides.

The peptide SIINFEKL itself, which requires no proteolytic processing before presentation, also showed enhanced presentation in the presence of UBC9, suggesting that UBC9 may regulate peptide transportation to ER lumen, and/or may affect antigen presentation steps in the ER.

5.1.2. Does UBC9 increase peptide translocation from the cytosol to the ER lumen? SIINFEKL co-translationally targeted to the ER with an ER signal sequence bypasses the standard antigen presentation cytosol-to-ER pathway, the TAP transporter, but even with this construct the H-2K^b-SIINFEKL levels on UBC9 transfected cells were somewhat higher than that on control vector transfected cells (Fig 15), although the effect is not as marked as in cytosolic minigene transfected cells. This suggested that UBC9 affects steps both in the cytosol (possibly TAP transport) and in the ER lumen,that occurs after peptides are generated. .

Taken together, these results suggest that multiple components of the antigen presentation pathway may be regulated by UBC9. UBC9 had the strongest effect on full length ovalbumin and the C-extended minigene, suggesting that UBC9 affects proteasome activity. However, UBC9 also increased H-2K^b-SIINFEKL level in ER-targeted SIINFEKL-transfected cells and in cells hypertonically loaded with SIINFEKL peptide, suggesting that UBC9 may regulate cytosolic and/or ER components as well. Immunofluorescence analysis confirmed these expectations. In UBC9-overexpressing cells (but not in their untransfected neighbors), several components of the immunoproteasome (LMP2, LMP7, and MECL1) were upregulated, which could account for the enhanced generation of SIINFEKL from proteasome-dependent precursors.

The LMP2 and LMP7 genes are located in MHC class II region (11), which first suggested that they might play a role in antigen presentation, though they were originally

found not required for MHC class I antigen presentation (10, 212). Upon IFNγ treatment, the expression of MECL1, LMP2, and LMP7 is induced and these subunits incorporate into proteasomes (producing a particle termed the "immunoproteasome") (13) (184, 185, 213). Immunoproteasomes have different peptidase activities from constitutive proteasomes, although the ubiquitin-dependent protein degradation rate is not changed. Immunoproteasomes have a preference for cutting proteins after hydrophobic and basic, but not acidic, amino acids, generating more peptides suitable for MHC class I binding (13). In vitro experiments showed that purified immunoproteasomes do generate more precursors of antigenic peptides, which favors MHC class I antigen presentation (29). In LMP2 and LMP7 knockout mice, MHC class I expression levels and CD8+ T cells are reduced, and immune response are impaired, suggesting that LMP2 and LMP7 play important roles in MHC class I antigen presentation and the immune response (28, 32, 33). Thus, the increase in immunoproteasomes stimulated by UBC9 may contribute to the large increase in presentation of antigens that require cleavage by proteasomes.

But effects on the proteasome can not account for all my findings, since UBC9 overexpression also increased presentation from proteasome-independent precursors. Immunofluorescence microscopy also revealed that cells expressing UBC9 expressed higher levels of TAP1 compared to their UBC9-negative neighbors, suggesting that UBC9 may facilitate peptide translocation from the cytosol into the ER lumen. This may explain why UBC9 overexpression increased H-2K^b-SIINFEKL level on cells loaded with N-terminal-extended SIINFEKL and SIINFEKL peptide, which are not dependent on the proteasome for proteolytic processing.

The TAP complex, which is involved in transporting peptides from the cytosol to ER lumen for MHC class I binding, is composed of two homologous proteins, TAP1 and TAP2, and helps to determine the peptides displayed on the cell surface and thereby affect T cell immune responses (214, 215). TAP levels are normally relatively low, but upon IFNγ treatment TAP expression is greatly upregulated (216), which in turn enhances peptide transportation from cytosol into ER lumen for MHC class I binding (217, 218).

Upregulation of TAP and the presence of immunoproteasomes are still not sufficient to explain the effect of UBC9 on ER-targeted SIINFEKL, since this precursor bypasses cytosolic proteolysis and TAP transport. These effects could be explained by the further finding that tapasin was increased in UBC9 transfected cells.

Tapasin associates with both TAP and the MHC class I heavy/β2-m complex in the ER, and increases MHC class I antigen presentation by increasing peptide binding through tethering TAP and MHC class I molecules together and even increasing TAP levels (219). As with TAP, LMP2, LMP7, and MECL-1, tapasin is constitutively expressed at low levels and can be up-regulated by several cytokines, especially IFNγ (220-222), which may enhance peptide binding in ER. The upregulation of tapasin could potentially account for the effects of UBC9 on presentation of SIINFEKL from ER-targeted precursors.

It is important to note that UBC9 overexpression did not increase the levels of leucine aminopeptidase, and that levels of the ER chaperones calnexin, calreticulin, ERp57 and PDI

(all of which interact with MHC class I during maturation in the ER) are not changed in UBC9-transfected cells. This is important for three reasons. First, this demonstrates the specificity of the immunofluorescence microscopy, making it unlikely that the increased intensity of TAP, tapasin, LMP2, LMP7, and MECL1 staining were nonspecific. Second, it suggests that UBC9 specifically affects some, but not all, of the steps in MHC class I antigen presentation pathway. Third, since LAP is interferon-inducible (48), this demonstrates that UBC9 does not entirely replicate the effects of interferon treatment, but rather must affect proteins through a separate mechanism.

IFNγ upregulates many genes associated with antigen processing, including those I show here are upregulated by UBC9, by increasing their transcription. Could UBC9 act by inducing IFNγ? This seemed unlikely, since UBC9 did not affect levels of LAP, which is also IFNγ-inducible. To further examine this possibility, I measured mRNA levels of MECL1 in cells overexpressing UBC9, using real-time PCR. Although the protein shows a marked increase by immunofluorescent microscopy (Figure 23A), there was no change in the mRNA level compared to control-transfected cells (Figure 24). This shows that UBC9 does not act through IFNγ, and further suggested that UBC9 may act post-transcriptionally.

However, directly measuring the effect of UBC9 overexpression on several of the proteins in the class I pathway proved technically difficult, for two reasons. First, the endogenous levels of the IFN-induced proteins MECL1, LMP2, and LMP7 are constitutively very low, and even TAP1 (which is constitutively expressed) is normally only present at low levels. Tapasin, was not detectable in COS-Kb cells, perhaps because of

species differences in these African Green Monkey cells. I was therefore unable to detect the endogenous proteins in immunoblots from COS-Kb cells, although RT-PCR, which is generally much more sensitive than immunoblotting, detected mRNA transcripts for these genes. As well, overexpressing UBC9 by transient transfection results in only a minority of cells overexpressing the gene: 20-30% of the total population in COS-Kb cells, and around 10% for Hela-Kb. Even if these cells strongly upregulated levels of a particular protein, in an immunoblot the increase would be difficult to detect against the background of untransfected cells. I used a vaccinia virus that expresses UBC9 to infect the COS-Kb cells, hoping that this would cause overexpression in a large proportion of the population. However, these COS-Kb cells are not highly infectable with vaccinia virus, and a majority of these cells consistently do not express high levels of recombinant genes from vaccinia virus [personal communication from Ian York].

I therefore performed co-transfection experiments, transiently transfecting COS-Kb with both UBC9 and LMP2, LMP7, or MECL1, and immunoblotted for the co-transfected protein. In these experiments most cells will express both of the costransfected genes. Immunoblots from these experiments showed marked increases in the level of LMP2, LMP7, MECL1, PA28α, and PA28β protein in the presence of UBC9, compared to control-transfected cells (Fig 25), and pulse-chase experiments showed that the proteins were much more stable in the UBC9-overexpressing cells than in the control-transfected cells (Fig 28).

When I performed similar experiments with human tapasin, UBC9 itself did not increase tapasin levels. However, UBC9 did increased tapasin level when SUMO-2 was

also co-transfected. This is consistent with the results that suggested that SUMO-2 may be involved in MHC class I antigen presentation. Tapasin is a very stable protein, and even with 8-hour chases we did not see any obvious protein degradation even in the absence of UBC9. I was therefore unable to measure changes in degradation of this protein.

In summary, my findings with the different SIINFEKL precursors suggested that UBC9 overexpression affected proteasome function, as well as ER components of the antigen presentation pathway. The observation that UBC9 overexpression induces immunoproteasome catalytic subunits, TAP, and tapasin provides an explanation for these findings. While it is possible that UBC9 overexpression upregulates other components of the antigen presentation pathway, these effects we have documented could explain the effects of UBC9 overexpression on antigen presentation.

5.1.3. How does UBC9 work?

Unlike other UBCs, which conjugate ubiquitin to target proteins for proteasome-dependent degradation, UBC9 conjugates SUMO molecules to target proteins (131). Sumoylation has multiple effects, including altering subcellular localization, protein stabilization, and transcriptional regulation (143, 147, 149, 150, 161, 209, 223, 224). As with ubiquitination, sumoylation is a multiple-step process in which the heterodimeric SUMO-activating enzyme (composed of SAE1 and SAE2 (165) and UBC9 transfer SUMO molecules to protein substrates (140).

To know whether UBC9-mediated sumoylation is involved in the regulation of MHC class I antigen presentation proteins, a mutant form of UBC9, UBC9OH, whose Cys93 has been changed to serine, was used to transfect COS-K^b cells. This mutant is unable to conjugate SUMO to substrate proteins (147). UBC9OH showed the same effect on antigen presentation as did wild-type UBC9, which initially seemed to to rule out the involvement of sumoylation in MHC class I antigen presentation regulation. Interestingly, several papers have reported that mutation of Cys93 in UBC9 does not affect its interaction with substrate proteins (164, 173, 174), even though most of the interacting proteins are sumo-1 substrates (163), suggesting UBC9 may regulate the function of its targets by direct interactions as well as, or instead of, by sumoylation. On the other hand, our SUMO transfection results (Fig 34) suggested that sumoylation may be involved in MHC class I antigen presentation regulation.

5.1.4. SUMO-2 may be involved in MHC class I antigen presentation regulation

In mammalian cells, there are at least three SUMO molecules: SUMO-1, SUMO-2 and SUMO-3. To study which, if any, SUMO molecule(s) is involved in the regulation of MHC class I antigen presentation, CD16-OVA-COS-K^b cells were transfected with wild-type and mutant SUMO molecules. Only wild type SUMO-2, but not mutant SUMO-2 and other SUMO molecules, increased the H-2K^b-SIINFEKL levels on the cell surface (Figur 34), suggesting that SUMO-2 regulates MHC class I antigen presentation.

To determine whether SUMO-2 works together with UBC9, I co-transfected COS-K^b cells with UBC9 and each of the three SUMO molecules, including for each wild-type and mutant forms in which the active conjugation sites were inactivated. If any SUMO molecule is involved in MHC class I antigen presentation regulation, its mutant form should abrogate its function (225). Wild-type SUMO-1 and SUMO-3 behaved the same as mutant SUMO-1 and SUMO3 in these experiments. However, wild-type and mutant SUMO-2 behaved differently in cooperating with UBC9. UBC9 and wild-type SUMO-2, but not UBC9 and mutant SUMO-2, increased H-$2K^b$-SIINFEKL levels on ovalbumin stable transfectants, suggesting that UBC9 ismediating its effects by conjugating SUMO-2 (Figure 33). These experiments suggest that SUMO-2 and SUMO1 and -3 have different functions in vivo, though they are highly homologous, and implicate SUMO2 as the relevant SUMO molecule in the regulation of MHC class I antigen presentation.

However, H-$2K^b$-SIINFEKL levels were always lower on cells co-transfected with both UBC9 and SUMO-2 than on cells transfected with either UBC9 and SUMO-2 independently: i.e. not only did we not see any additive effect of SUMO-2 and UBC9, co-transfection slightly reduced the effect. (These results are summarized in Table 3). If they are working through each other, I would predict that they should result in an additive/synergistic effect. One possible explanation for the reduced effect in double-transfected cells is that in the double transfections, the expression levels of both SUMO-2 and UBC9 are lower than single transfections. In fact, in double transfection experiments, the UBC9 and SUMO expression levels were each much lower than single transfection with either construct alone (data not shown).

Although UBC9OH has the same effect as wild type UBC9 on MHC class I antigen presentation, there was no effect when COS-K^b cells were co-transfected with both mutant UBC9 and mutant SUMO-2. However, when mutant UBC9 was co-transfected with wild type SUMO-2, H-$2K^b$-SIINFEKL levels were increased (summarized in Table 3). Again, these experiments suggest that SUMO-2 and UBC9 affect the same step in MHC class I antigen presentation pathway.

When I co-transfected MECL1, LMP-2, and LMP-7 (as potential substrates for sumoylation) with UBC9 and SUMO molecules, I failed to detect any sumoylated protein. However, it is technically difficult to detect sumoylated proteins in extracts of cells, since highly-active isopeptidases in cell lysates may rapidly remove SUMO molecules from their conjugates ; therefore my inability to show any sumoylation does not mean the proteins are not, in fact, sumoylated.

5.1.5. Model for UBC9 regulating MHC class I antigen presentation

UBC9 overexpression clearly upregulates MHC class I antigen presentation. This is accomplished at least partly by altering the activity of proteasomes, by increasing LMP2, LMP7, and MECL-1, which incorporate into proteasomes and alter the catalytic activity in such as way as to increase the production of antigenic peptides and their precursors. BH levels may also be increased, which may help to trim antigenic peptides. UBC9 overexpression also increases TAP1 and tapasin levels, which will increase transport of

peptides into the ER and enhance their association with newly-synthesized MHC class I molecules. The effect of UBC9 is mediated, at least for some of these proteins, by stabilizing the protein and reducing degradation.

The simplest explanation for UBC9's effect on these proteins is that it is mediated directly by sumoylation. Unlike ubiquitination, it is difficult to generalize the effects of sumoylation on proteins. For transcription factors, sumoylation always seems to repress their activity (158, 226, 227) (228). Subcellular localizationis another important but distinct function of sumoylation. The best example for this is cytosol-nuclear transport (130, 229). I tested whether UBC9 causes ovalbumin to be transported to specific subcelluar localizations, such as PML oncogenic domains (PODs), which accumulate immunoproteasomes and PA28 upon IFNγ treatment (230) and are therefore candidates for antigen processing; however, I did not find that ovalbumin was co-localized with PML protein (data not shown). Our minigene transfection and peptide loading experiments also suggested that UBC9 does not regulate MHC class I antigen presentation by sumoylation of the antigen, since SIINFEKL, whose presentation is enhanced by UBC9, is very unlikely to be a SUMO substrate.

Sumoylation with SUMO1 has been shown to stabilize some proteins (161). The mechanism for this stabilization is not clear. In some cases (e.g. when SUMO1 is conjugated to I-κBα), it seems that the SUMO molecules are conjugated to the same lysines that would otherwise be used by ubiquitin, and by blocking ubiquitination proteasome-mediated degradation (161). UBC9 overexpression increases the levels of several MHC

class I pathway-associated proteins. Since we were unable to detect sumoylation of these proteins, it is difficult to say whether sumoylation of these proteins was blocking ubiquitination.

UBC9OH, a mutant of UBC9 that is unable to conjugate SUMO to substrates, increases MHC class I antigen presentation as well as wild-type UBC9, which argues that UBC9 increases substrate levels by direct interaction rather than sumoylation. However, mutant SUMO-2 transfection, in contrast to wild-type SUMO-2, faiiled to increase MHC class I antigen presentation, suggesting that SUMO molecule is involved in UBC9 function in MHC class I antigen presentation. This raises a more complicated model where UBC9OH might be working in concert with endogenous wild type UBC9 or some other UBC. For example, UBC9OH may dock to substrates only after a SUMO-2 molecule is already attached (which in our system would be conjugated by the endogenous wild-type Ubc9) (Fig 46).

In normal cells, few proteins are conjugated to SUMO2 and 3 in the steady state (172), although in stressed cells SUMO2 and 3 are rapidly conjugated to high-molecular-weight proteins (172). It is not known whether the low level of conjugation found in normal cells is because the SUMO molecules are not conjugated, or because they are conjugated and then rapidly removed from target proteins by desumoylating enzymes (231, 232). The presence in normal cells of large amounts of isopeptidases that efficiently remove SUMO molecules from their substrates (233) suggests that the latter may be the case. This provides a possible explanation for two points. First, according to my model above, UBC9 or UBC9OH must

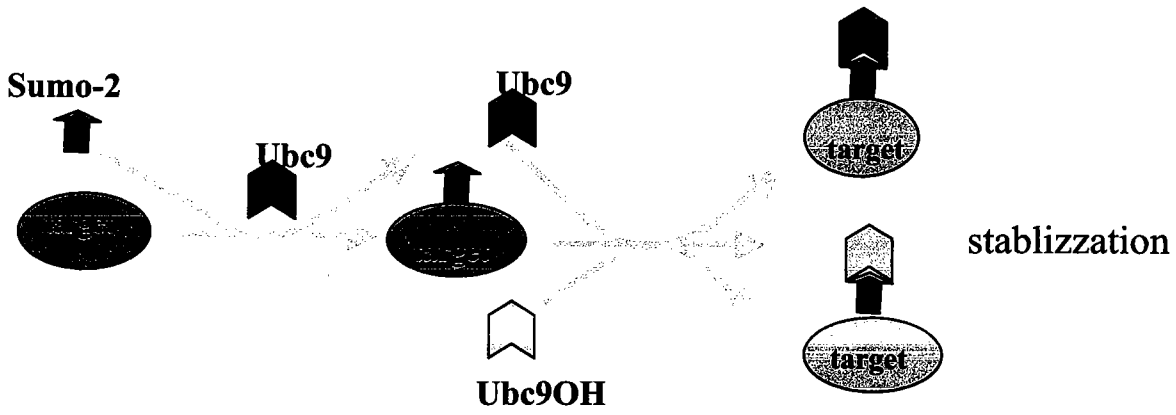


Fig 46. Model for UBC9 stablize target proteins. Either overexpressed or endogenous UBC9 conjugate SUMO-2 to target protein. The SUMO-2 conjugated proteins can be physically associated by either wildtype or catalytically inactive UBC9, which stablize the target proteins.

interact with sumoylated proteins; yet I and others have found little evidence for sumoylation of these or other proteins. If proteins are sumoylated transiently, then UBC9 (or UBC9OH) could interact with the proteins and alter their function during their sumoylated phases. Second, I found that overexpressing SUMO2 (but not mutant SUMO2 that cannot be conjugated to substrates) enhances antigen processing. If SUMO2 is not normally conjugated to substrates, it is difficult to account for this, since overexpressing SUMO2 would simply increase the pool of free SUMO2. However, if there is normally constant sumoylation and desumoylation, then increasing the pool of free SUMO2 by transfection should push the equilibrium toward conjugation, effectively increasing the sumoylated phase for substrate proteins, and increase the period during which endogenous or transfected UBC9 could interact with the proteins. According to this reasoning, mutant SUMO2, which is unable to interact with substrate proteins, would not enhance antigen processing; this is what we observed.

Finally, if cells are co-transfected with both mutant UBC9 and mutant SUMO-2, UBC9OH will not conjugate the endogenous wild-type SUMO-2, and transfected mutant SUMO-2 will compete for the endogenous wild-type UBC9 with endogenous wild-type SUMO-2 for conjugation to target proteins. As a result, target proteins will not be sumoylated, and neither the wild-type nor the mutant UBC9 will interact with the target proteins.

Although, at least in COS cells, there are high level of SUMO-2 and SUMO-3 molecules, their function in vivo is not known. My in vivo transfection experiments suggest that SUMO-2 may be involved in protein degradation and stablization.

While many proteins are regulated by sumoylation, there are also examples of proteins that are apparently regulated by direct interaction with UBC9, including the DNA repair proteins RAD51 and RAD52 (132), cell cycle regulatory factors CLB2 and CLB5 (an S-phase cyclin) (123), and the glucocorticoid receptor (147). Interestingly, many of these substrates are also found to be sumoylated. For example, it was found that glucocorticoid receptor is post-translationally modified by SUMO-1 (234, 235). Since I found that overexpression of UBC9OH (which cannot conjugate SUMO to target proteins) affects antigen presentation to the same extent as UBC9, it is also possible that Ubc9 is working in our system fully or in part independently from sumoylation.

5.1.6. UBC9 knockout does not decrease H-2K^b-SIINFEKL level

How physiologically significant is UBC9's effect on MHC class I antigen presentation? I attempted to addresse this question with siRNA, which has been used to knock out many genes in vivo (203-205, 206, York, 2002 #596). I successfully knocked out UBC9 with siRNA, but when UBC9 was eliminated, the cells stopped growing, and plasmid transfection efficiency was very low. These results suggested that UBC9 is essential for mammalian cell viability, which is consistent with recent observations (236). Elimination of UBC9 had no effect on presentation of full-length ovalbumin, but increased presentation

of ER-targeted SIINFEKL and of MSIINFEKL. One would expect elimination of UBC9 to have opposite effects to overexpression, but both treatments increased antigen presentation. It seemed possible that overexpression of UBC9 had a dominant-negative effect, which would lead to the same effect as siRNA-mediated elimination. However, if this was the case, the siRNA results should precisely mirror those of the overexpression studies, while in fact not all the siRNA results were consistent with my UBC9 overexpression experiments, in which UBC9 has the strongest effect on full-length ovalbumin, but had relatively little effects on SIINFEKL minigene and ER targeted SIINFEKLminigene.

Because elimination of UBC9 induced cell death, these studies are not definitive, and the results may reflect non-specific effects of cell stress or death. However, the lack of an effect on full-length ovalbumin when UBC9 is eliminated is generally consistent with the model presented above. Since cells normally express very low levels of immunoproteasome components, reducing them (as might happen if UBC9 is eliminated) should not affect ovalbumin processing. It is also possible that UBC9 knockout decreases the generation of suitable peptides, which may give ER-targeted or minigene-generated SIINFEKL more chance to bind to MHC class I molecules by reducing competition. In fact, I did find that UBC9 knockout increased ER-targeted SIINFEKL and MSIINFEKL, but not full-length ovalbumin, presentation (Fig 30). However, the overall MHC class I expression level was increased in UBC9 knockout cells, implying that generation of other peptides was also increased (rather than reduced). It is possible that UBC9 knockout leads to cell stress or other non-specific effects which may increase MHC class I antigen presentation.

It is not possible to fully study the function of UBC9 on MHC class I antigen presentation by knocking out UBC9, since UBC9 seems to be critical for cell viability. One alternative is to find the SUMO conjugation sites of targeted proteins. By comparing the SUMO conjugation site sequence of UBC9 target proteins, Sampson et al. found the consensus sequence of SUMO-1 conjugation is PsiKXE, in which Psi stands for a large hydrophobic amino residue, and X stands for any amino acid (237). By mutating the conjugation sites, it may be possible to study the function of UBC9 on MHC class I antigen presentation. UBC9 may also increase protein levels via an indirect effect. For example, within MECL-1, LMP2 and LMP7, UBC9 may increase any one of them, which may stabilize the other two subunits by increasing incorporation into immunoproteasomes; or UBC9 may increase TAP1 levels by increasing tapasin (219). The fact that UBC9OH increases K^b-SIINFEKL level also hints that sumoylation might not be not essential. More work is needed to find the mechanism by which UBC9 increase MHC class I antigen presentation.

5.1.7. Biological significance.

While little is known about the effects of modification with SUMO-2 and -3, it is likely that their targets and functions are different from SUMO-1. For example, SUMO-1 is predominately found conjugated to nuclear membrane proteins, while SUMO-2-conjugated proteins are located in the nuclear bodies and SUMO-3-conjugated proteins are mainly found in the cytosol (238). Unlike SUMO-1, which is constitutively mainly found conjugated to proteins, a large pool of free SUMO-2 and SUMO-3 can be found. When

cells are stressed by heat shock or oxidative stress, however, SUMO2 and 3 rapidly become conjugated to high-molecular-weight proteins (172, 238).

One source of cell stress is virus infection, which is often associated with massive viral protein accumulation and induction of the unfolded protein response (239, 240). The unfolded protein response causes, among other things, a rapid and potent inhibition of translation of cellular proteins, and many viral infections also directly inhibit host protein synthesis through other means. Under these circumstances, therefore, when an immune response would be most appropriate, new synthesis of proteins required for antigen processing would be inhibited.

We speculate that under conditions of cell stress, UBC9 conjugates SUMO2 or SUMO3 to proteins associated with antigen processing. These sumoylated proteins are degraded much less rapidly than unconjugated versions, so that levels of these proteins are relatively spared and the cell can maintain antigen presentation even while other protein levels are reduced. If the cellular stress was in fact the result of viral infection, then recognition of the infected cells by CTL will result in IFNγ expression and infected cells may also release type II IFNs. These cytokines induce transcriptional upregulation of genes involved in antigen processing, so that the infected cell can enter a fully-effective antiviral mode. It has been reported that IFNγ also induces UBC9 expression (208) (although I did not find the increased UBC9 protein expression level), so that IFNγ treatment may also help to stabilize pre-existing proteins as well as induce synthesis of new ones. According to this model,

sumoylation is a rapid response to cell stress that maintains antigen presentation even while overall protein expression is reduced.

My experiments showed that UBC9 overexpression regulates MHC class I antigen presentation. When using ovalbumin as a model antigen, UBC9 overexpression markedly increases the levels of the immunodominant epitope SIINFEKL on the cell surface. However, in most of the cells I examined, the total level of MHC class I on the cell surface did not change nearly as much, implying that peptide supply in general was not increased to the same extent as SIINFEKL. This in turn suggests that UBC9 effects on antigen presentation may actually inhibit the presentation of some epitopes, rather than universally enhancing them.

5.2. HSP27 overexpression inhibits MHC class I antigen presentation

HSP27 is expressed in a variety of tissues in the absence of stress and is involved not only in apoptosis, such as virus-induced cell death (85), but also in cell survival, such as in injured sensory and motor neuron survival and in TNF-mediated apoptosis (86, 87). Human HSP27 is a potent inducer of IL-10 in human monocytes, suggesting a possible role in immune responses (88). HSP27 expression has been shown to confer resistance to cell death by interfering with apoptosis in response to multiple stresses (86, 241, 242). Phosphorylated HSP27 has been shown be involved in cytoskeletal rearrangements, suggesting that HSP27 is involved in cell movement or particle transportation within cell (243, 244).

While studying UBC9's mechanism of action, I examined some proteins that are known to interact with UBC9. In Drosophila, HSP27 has been shown to interact with UBC9 (210). Based on the high sequence homology between mammalian and Drosophila UBC9 (83% identity), we expected that mammalian UBC9 would also interact with HSP27. When a protein of approximately the right molecular weight was found to be conjugated to SUMO in the presence of UBC9 overexpression (even though this later proved to be UBC9 itself, I therefore asked whether HSP27, like UBC9, affects MHC class I antigen presentation. HSP27 overexpression markedly reduced H-2K^b-SIINFEKL levels when COS-K^b cells were hypertonically loaded with ovalbumin protein. As well as showing that HSP27 overexpression inhibits MHC class I antigen presentation, these results suggested that HSP27 does not inhibit MHC class I antigen presentation by inhibiting protein synthesis. HSP27 overexpression inhibited MHC class I antigen presentation in E36-K^b cells transfected with plasmids expressing many forms of SIINFEKL precursors, including full-length ovalbumin, ER-targeted SIINFEKL, and N-extended and C-extended SIINFEKL, as well as MSIINFEKL. The effect on ER-targeted SIINFEKL seemed particularly significant, since this construct targets SIINFEKL directly into the ER lumen, while HSP27 is a cytosolic protein. The results with this construct show that HSP27 does not affect peptide transportation from the cytosol to the ER via the TAP complex. It is also very unlikely that HSP27 inhibits MHC class I antigen presentation by inhibiting protein degradation, since constructs that are dependent on different proteolytic pathways (i.e. the proteasome and aminopeptidases) were all affected. It seems that HSP27 affects steps of

MHC class I antigen presentation that are in the ER or after the ER, e.g. protein transportation to the cell surface.

When COS-7 cells were co-transfected with HSP27 and H-2K^b or H-2D^b, the levels of both H-2K^b and H-2D^b were lower than on cells transfected with control vector (Fig 45). However, HSP27 overexpression did not decrease surface expression of the human MHC class I allele HLA-A3 (Fig 39, 45), suggesting that the effects of HSP27 on MHC class I antigen presentation differ depending on the MHC class I allele. As well, Hsp27 overexpression decreased levels of influenza hemagglutinin on cell surface, suggesting HSP27 inhibits transportation of some membrane proteins, rather than specifically affecting MHC class I.

HSP27 is a phosphorylated protein, in which there are three potential phosphorylation sites: S15, S78 and S82. To know whether HSP27 phosphorylation is involved in regulating MHC class I antigen presentation, point mutations were introduced to generate S15A, S78A and S82A. None of these mutations affected HSP27's effect on MHC class I antigen presentation, suggesting that protein phosphorylation is not involved in HSP27 function in MHC class I antigen presentation. However, since I did not make double or triple mutations, I can not rule out the possibility that these phosphorylation sites have redundancy in regulating HSP27 function.

Since UBC9 has been reported to interact with HSP27, we speculated that UBC9 may increase MHC class I antigen presentation by conjugating SUMO-2 molecules to HSP27

and inhibit its function. However, I failed to find sumoylated HSP27 protein by immunoprecipitation or immunoblotting. Also, after introducing point mutations to HSP27, in which all potential sumoylation sites were individually mutated from lysine to serine, there was no difference in HSP27's effect on MHC class I antigen presentation. These findings suggested that UBC9 does not regulate MHC class I antigen presentation by sumoylating HSP27. However, again, since I did not mutate all the lysines simulataneously, it is possible that sumoylation of HSP27 is redundant, and that more than one lysine can be sumoylated with the same effect. It is also possible that HSP27 inhibits MHC class I antigen presentation only when it is not sumoylated, and that overexpressing even wild-type HSP27 overwhelms the sumoylation machinery and results in excess unsumoylated HSP27. In that case, mutating the sumoylation sites would not affect the inhibition.

HSP27 exists in vivo as multimers ranging from dimers to >700-kDa oligimers. Upon phosphorylation, HSP27 oligomers are dissociated and regulate actin filaments by association. HSP27 plays a very significant function in regulation of the actin cytoskeleton (245). In fact in vitro experiments showed that unphosphorylated HSP27 inhibited actin polymerization (246). It is possible that this mechanism might account at least in part for HSP27s effect on antigen presentation: HSP27 overexpression might alter cytoskeleton formation, which might play a role in the transport of membrane proteins to the cell surface.

HSP27 knockout inhibits MHC class I antigen presentation

To know the physiological importance of HSP27 in MHC class I antigen presentation in vivo, we used siRNA to knock down HSP27. HSP27 was successfully knocked down by

at least 95% in COS-K^b cells (Fig 43). Surprisingly, HSP27 knockdown, as with overexpression, impaired MHC class I antigen presentation. HSP27 knockdown decreased not only generation of H-$2K^b$-SIINFEKL complexes from all full-length ovalbumin and SIINFEKL peptide precursors, but also overall H-$2K^b$ levels, suggesting the significance of HSP27 in MHC class I antigen presentation.

Therefore both overexpression and reduction of HSP27 expression had a negative effect on MHC class I antigen presentation. In the absence of HSP27, the cytoskeleton may be not regulated properly, which may also affect protein transport to the cell surface. However, this would not be consistent with our finding that the surface expression of HLA-A0302 was not affected by the loss or overexpression of HSP27. It is noteworthy that (as with UBC9 siRNA) when HSP27 was knocked out, the cells became sick and stopped growing, suggesting that HSP27 is essential for cell viability, which may be related to its function in modulating cytoskeletal functions. HSP27 deficiency results in death of embryonic stem cells (247). The apparent requirement for HSP27 for cell viability means the siRNA results should be interpreted with caution.

How does HSP27 affect MHC class I antigen presentation? As I noted above, since HSP27 overexpression affects the presentation of SIINFEKL from ER-targeted SIINFEKL, it must affect some steps after peptides have been transported into ER,rather than the peptide-generation aspects of the pathway. This possibility was supported by the finding that HSP27 decreased influenza virus hemagglutinin expression at the cell surface. I propose that HSP27 may regulate MHC class I complex transportation by regulating the

cytoskeleton. It is possible that when HSP27 is over-expressed, it acts as a dominant negative form (perhaps because it overwhelms the sumoylation machinery, and only the sumoylated form is functional), which confers a very similar phenotype to HSP27 knockdown.

Several heat shock proteins have been reported to be involved in MHC class I antigen presentation, including Hsp70 and gp96 (79, 80). A role for HSP27 in MHC class I antigen presentation has not been previously reported. Unlike other HSPs, HSP27 is not stress-inducible (84). Although it has been reported that Drosophila UBC9 interacts with HSP27, I did not find human UBC9 interact with HSP27 in my immunoprecipitation experiments. This may be further studied by a yeast two-hybrid approach. But it is very unlikely UBC9 regulates MHC class I antigen presentation via regulating HSP27. Since HSP27 inhibit all SIINFEKL precursors presentation, and influenza HA surface expression, HSP27 seems inhibit protein traveling from ER to cell surface

What is the biological significance?

The biological significance of HSP27 overexpression remains unclear. The effect of HSP27 overexpression on HA suggests that it is not specific for the immune system, but rather may play a general role in protein transport. The fact that HSP27 siRNA-treated cells are not viable supports this possibility. The observation that overexpression of HSP27 does affect the expression of some MHC class I alleles may be relevant if, in normal cells, HSP27 levels are functionally regulated. The connection to sumoylation (described by D. Joanisse (210), although in our experiments we were unable to detect sumoylated HSP27) suggests

one possible means for regulating HSP27, if either only sumoylated, or only non-sumoylated, HSP27 is functional. My finding that, in general, UBC9 and SUMO-2 enhance antigen presentation leads to the prediction that non-sumoylated HSP27 may inhibit MHC class I expression on the cell surface. In that case, one of the ways through which UBC9 overexpression enhances antigen presentation would be by inhibiting HSP27's inhibition of surface transport. Since cell stress does not directly regulate HSP27 levels (85, 87)UBC9 may provide a link between certain forms of cell stress and functional regulation of HSP27.

It is also possible that HSP27 can be more directly regulated. For example, when macrophages were infected with Mycobacterium tuberculosis, HSP27 was down-regulated (248). This may represent a mechanism by which infected cells regulate antigen presentation in response to an intracellular pathogen. Conversely, HSP27 overexpression, which reduces the expression of some MHC class I alleles, may be a mechanism for immune evasion by tumors and viruses. In fact, many cancer and tumor cells have been reported to express high levels of HSP27 (249, 250). It will be of interest to examine the role of this HSP27 overexpression on antigen presentation in these cells. As well, examining HSP27 levels in cells infected by various viruses may help explain the role of this protein in antigen presentation.

Acknowledgement:

I will thank Dr. Elizabeth J. Luna for providing me pTracer-CMV and pTre2 vectors. Dr. Yan Shi helping me in HPLC, Dr. Lian-Jun Shen for providing mice bone marrow derived dendratic cells.

Appendix

The following are some recipes for some solutions used in my experiments.

Loading buffer:

First mix:

6.85 g sucrose

0.4 ml 1 M Hepes

in 30ml RPMI1640.

heat to 37°C. Then melt 2 bottles of PEG (2mg/bottle) at 56°C, and add to the above. Fill up to 40 ml with RPMI1640 and filter through 0.22μm.

Hypotonic medium:

60% (v/v) RPMI1640, no additive

40% (v/v) ddH_2O

Filter through 0.22μm.

SP-Optimem

Optimem-500 ml bottle (Gibco BRL 31985)

5 ml nutridoma (Boehringer Manheim 1011370)

27μl 1 M 2-ME

10ml L-Glutamine

5 ml Pen Strep

HCM

in 500ml RPMI1640, supplemented with 25 λ of 1M 2-Me, 10 ml of L-Glutamine, 5 ml of Non-essential AA, 5 ml PSF, 5 ml of HEPES, 50 ml of FBS.

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